PATENT

Application No. 10/821,710
Page 1

IPEW #

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Inventors: Michael Wayne GRAHAM *et al.*

Confirmation No.: 1697

Application No.: 10/821,710

Group Art Unit: 1635

Filed: April 8, 2004

Examiner: Schnizer, Richard A.

For: **Control of Gene Expression**

Commissioner for Patents
U.S. Patent and Trademark Office
Customer Service Window, **Mail Stop Amendment**
Randolph Building
401 Dulany Street
Alexandria, VA 22314

THIRD PARTY SUBMISSION UNDER C.F.R. 1.99 IN PUBLISHED APPLICATION

Sir:

This third party submission is being made under the provisions of 37 C.F.R. 1.99 for the purpose of calling the Examiner's attention to prior art considered relevant to the above U.S. Appl. No. 10/821,710 (published as US 2004/0237145 A1 on November 25, 2004).

The patent/publications submitted for consideration are listed below:

1. US 5,578,716
2. US 5,578,716 (redacted)
3. US 5,631,148
4. US 5,631,148 (redacted)
5. US 6,506,559
6. US 6,506,559 (redacted)
7. WO 94/01550
8. WO 94/01550 (redacted)
9. WO 97/11170
10. WO 97/11170 (redacted)

DKT scan
M R Schnizer
3/12/07
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03/07/2007 SZEWDIE1 00000043 501283 10821710
01 FC:1464 130.00 DA
02 FC:1806 180.00 DA

PATENT

Copies of the above listed patents and publications are attached hereto. The attachments are all in the English language. Redacted versions of several references are included, as permitted according to MPEP 1134.01(II). Redacted versions of WO 97/11170 and US 5,631,148, already of record, are also being submitted.

A copy of this submission with attachments is being served on the applicants by first class mail concurrent to this filing, at:

Patton Boggs LLP
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McLean VA, 22102

This submission is being filed after two months following the date of publication of the application. However, it is respectfully submitted that the consideration of the redacted references appears to now be appropriate in light of recent prosecution and therefore the references could not have been submitted within the two month time frame. The processing fee of $130.00 in accordance with Rules 1.99(e) and 1.17(i) is included. In addition, the required fee ($180.00) pursuant to Rules 1.99(b)(1) and 1.17(p) is included. Please charge the required fees totaling $310.00 to Deposit Account No. 50-1283.

A self-addressed postcard is attached so that the receipt of this submission might be acknowledged.

Respectfully submitted,
Cooley Godward Kronish LLP

Date: March 6, 2007

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THIRD PARTY SUBMISSION (Use several sheets if necessary) PTO Form 1449	Attorney Docket No.	Application No. 10/821,710
	Applicants: Michael Wayne GRAHAM *et al.*	**PAGE 1 of 1**
	Filing Date: April 8, 2004	**Group Art Unit:** 1635

U.S. PATENT DOCUMENTS

Initial		Document No.	Date	Name	Class	Sub-Class	Filing Date
	1.	US 5,578,716	11/26/1996	Szyf *et al.*			
	2.	US 5,578,716 (redacted version)	11/26/1996	Szyf *et al.*			
	3.	US 5,631,148	05/20/1997	Urdea			
	4.	US 5,631,148 (redacted version)	05/20/1997	Urdea			
	5.	US 6,506,559	01/14/2003	Fire *et al.*			
	6.	US 6,506,559 (redacted version)	01/14/2003	Fire *et al.*			

FOREIGN PATENT DOCUMENTS

		Document No.	Date	Country	Class	Sub-Class	Translation
	7.	WO 94/01550 A1	01/20/1994	WIPO			
	8.	WO 94/01550 A1 (redacted version)	01/20/1994	WIPO			
	9.	WO 97/11170 A1	03/27/1997	WIPO			
	10.	WO 97/11170 A1 (redacted version)	03/27/1997	WIPO			

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, etc.)

Examiner	Date Considered

Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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United States Patent [19]

Szyf et al.

[11] **Patent Number: 5,578,716**

[45] **Date of Patent: Nov. 26, 1996**

[54] **DNA METHYLTRANSFERASE ANTISENSE OLIGONUCLEOTIDES**

[75] Inventors: **Moshe Szyf**, Cote St. Luc, Canada; **Eric von Hofe**, Wellesley, Mass.

[73] Assignees: **McGill University**, Canada; **Hybridon, Inc.**, Worcester, Mass.

[21] Appl. No.: **161,673**

[22] Filed: **Dec. 1, 1993**

[51] **Int. Cl.**[6] **C07H 21/00**; A61K 48/00

[52] **U.S. Cl.** **536/24.5**

[58] **Field of Search** 514/44; 536/24.5

[56] **References Cited**

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Razin and Szyf, *Biochim. Biophys. Acta*, 782:331–342 (1984) "DNA Methylation Patterns Formation and Function".
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Szyf et al., *J. Biol Chem.*, 260:8653–8656 (1985) "Cell Cycle–dependent Regulation of Eukaryotic DNA Methylase Level".
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Feinberg and Vogelstein, *Nature*, 301:89–92 (1993) "Hypomethylation distingishes genes of some human cancers from their normal counterparts".
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Primary Examiner—Jacqueline M. Stone
Assistant Examiner—D. Curtis Hogue, Jr.
Attorney, Agent, or Firm—Hale and Dorr

[57] **ABSTRACT**

The invention encompasses tumorigenicity-inhibiting antisense oligonucleotide sequences complementary to mRNA or double-stranded DNA that encodes mammalian DNA methyl transferase. It further encompasses methods for inhibiting tumorigenicity and pharmaceutical composition comprises the tumorigenicity-inhibiting antisense nucleotide.

4 Claims, 4 Drawing Sheets

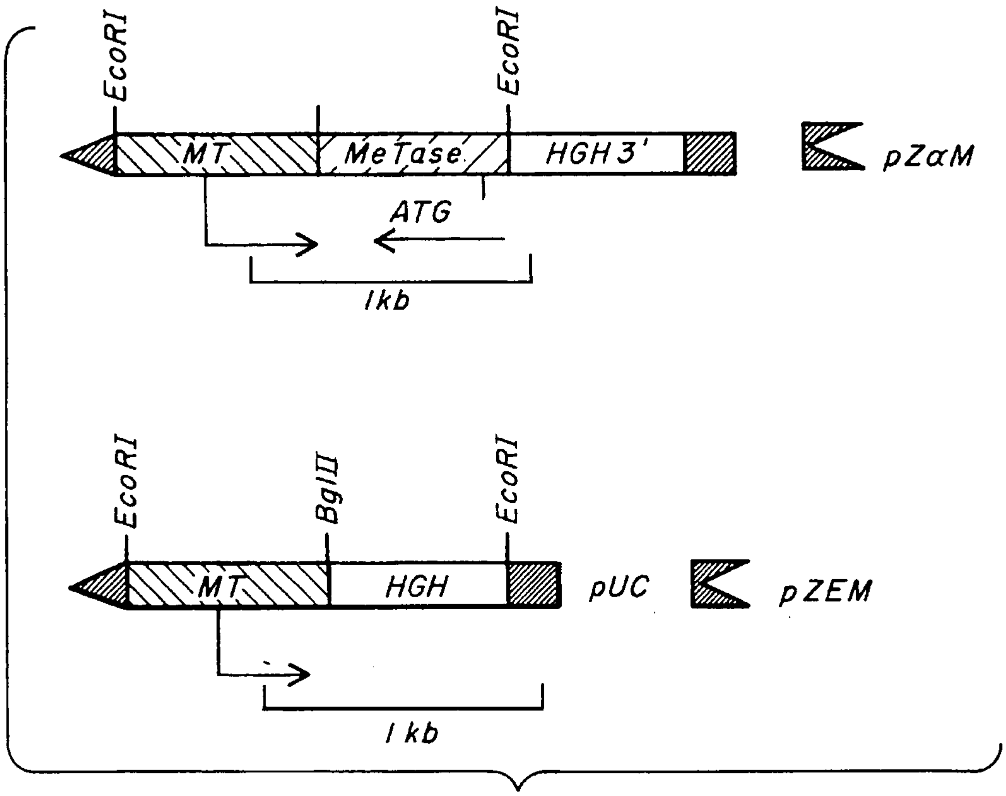
EcoRI
EcoRI
MT
MeTase
HGH 3'
pZαM
ATG
1kb
EcoRI
BglII
EcoRI
MT
HGH
pUC
pZEM
1 kb

FIG. 1

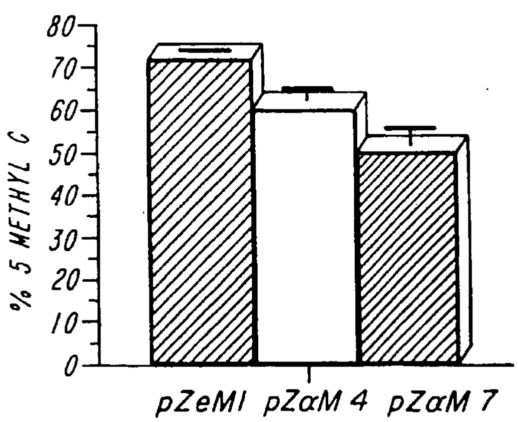
80
70
60
50
40
30
20
10
0
% 5 METHYL C
pZeM1
pZαM 4
pZαM 7

FIG. 2

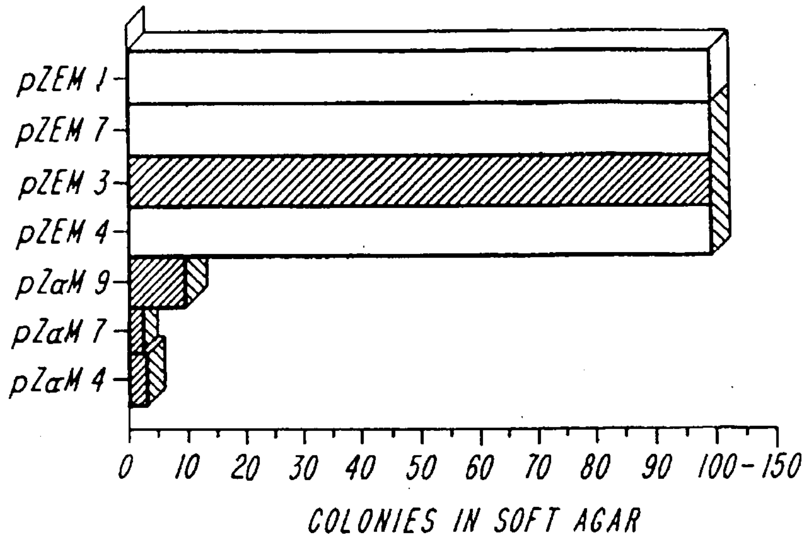
pZEM 1
pZEM 7
pZEM 3
pZEM 4
pZαM 9
pZαM 7
pZαM 4
0
10
20
30
40
50
60
70
80
90
100-150
COLONIES IN SOFT AGAR

FIG. 3

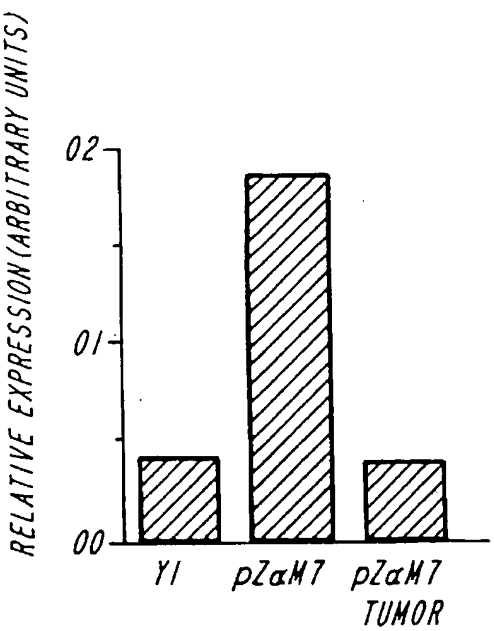
RELATIVE EXPRESSION (ARBITRARY UNITS)
02
01
00
Y1
pZαM7
pZαM7
TUMOR

FIG. 4

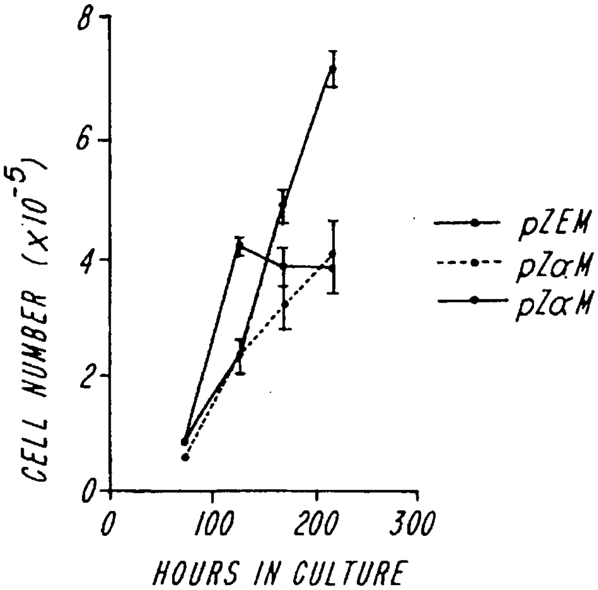
CELL NUMBER $(\times 10^{-5})$
8
6
4
2
0
0
100
200
300
HOURS IN CULTURE
pZEM
pZαM
pZαM

FIG. 5A

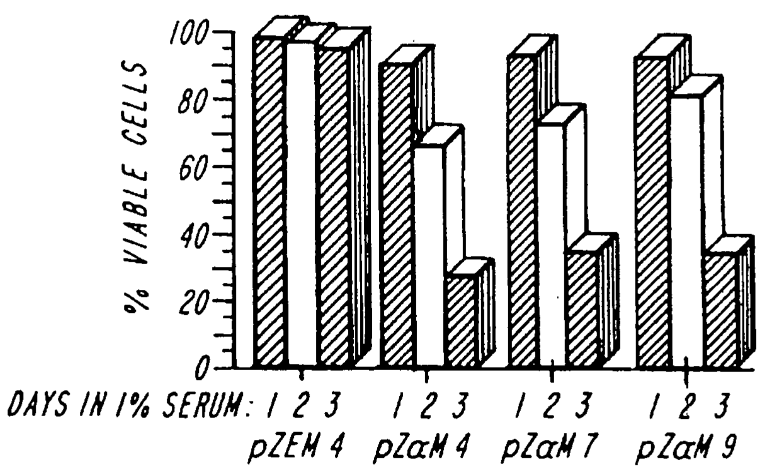
% VIABLE CELLS
100
80
60
40
20
0
DAYS IN 1% SERUM: 1 2 3
1 2 3
1 2 3
1 2 3
pZEM 4
pZαM 4
pZαM 7
pZαM 9

FIG. 5B

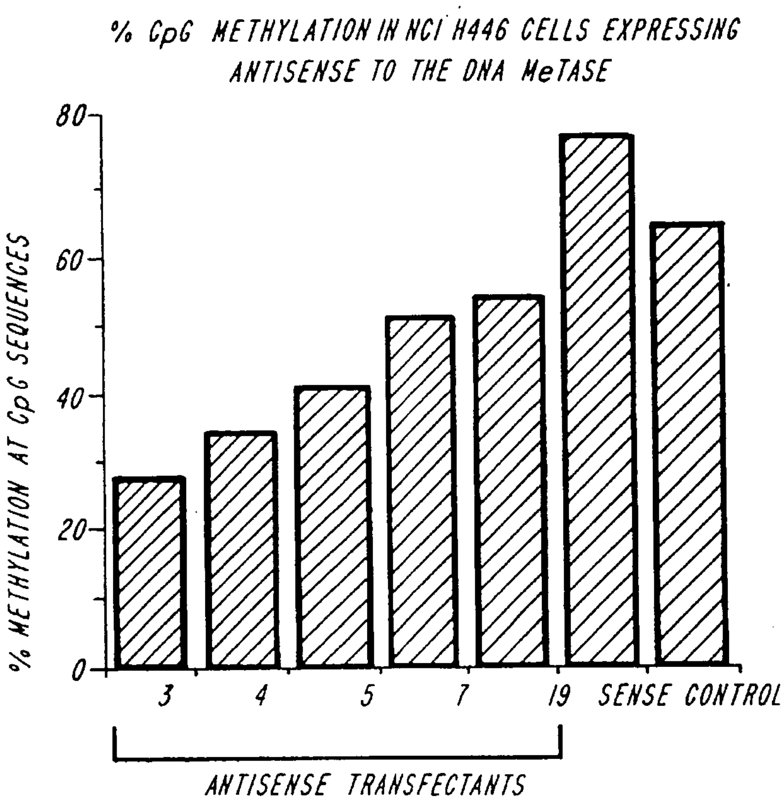
% CpG METHYLATION IN NCI H446 CELLS EXPRESSING
ANTISENSE TO THE DNA MeTASE
% METHYLATION AT CpG SEQUENCES
80
60
40
20
0
3
4
5
7
19
SENSE CONTROL
ANTISENSE TRANSFECTANTS

FIG. 6

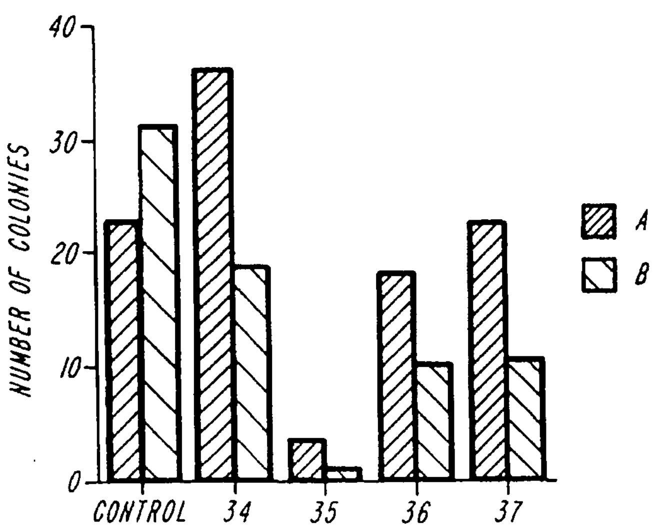
NUMBER OF COLONIES
40
30
20
10
0
CONTROL
34
35
36
37
A
B

FIG. 7

DNA METHYLTRANSFERASE ANTISENSE OLIGONUCLEOTIDES

FIELD OF THE INVENTION

This invention relates to oligonucleotides for use in the inhibition of DNA methyl transferase expression, and more particularly, antisense inhibition of tumorigenicity.

DESCRIPTION OF RELATED ART

Alterations in the normal gene expression profile of a cell are thought to be early events in oncogenic transformation. A large number of oncogenes are transcription factors. However, many oncogenes are not transcription factors but are involved in signal transduction pathways that trigger activation of transcription factors such as the activation of Jun by the RAS signalling pathway.

The DNA methyltransferase (DNA MeTase) gene 5' region has recently been characterized (Rouleau et al, *J. Biol. Chem.*, 267: 7368–7377 (1992)). It contains at least two functional AP-1 sites and the promoter of that gene can be dramatically transactivated by Fos, Jun or Ras. The DNA MeTase gene encodes an activity that is responsible for methylating cytosine residues in the dinucleotide sequence CpG. A hallmark of DNA methylation is that 80% of the CpG sites are methylated in a nonrandom manner generating a site-, tissue- and gene-specific pattern of methylation. Methylation patterns are formed during development. Establishment and maintenance (Razin and Szyf, *Biochim. Biophys. Acta*, 782: 331–342 (1984)) of the appropriate pattern of methylation is critical for development (Li et al., *Cell*, 69: 915–926 (1992)) and for defining the differentiated state of a cell (Szyf, et al., *J. Biol. Chem.*, 267: 12831–12836 (1992)). The pattern of methylation is maintained by DNA MeTase at the time of replication (Szyf et al., *J. Biol Chem.*, 260: 8653–8656 (1985)); the level of DNA MeTase activity and gene expression is regulated with the growth state of different primary and immortal cell lines (Szyf et al., *J. Biol. Chem.*, 266: 10027–10030 (1991)).

The relationship of DNA methylation to tumorigenicity has been in a state of confusion for some time. Some reports have suggested that hypomethylation of certain genes may be implicated in neoplasia (see e.g., Ohtani-Fukita et al., *Oncogene*, 8: 1063–1967 (1993)). However many reports have demonstrated hypomethylation of total genomic DNA (see e.g., Feinberg et al., *Cancer Res.*, 48: 1159–1161 (1988); Goelz and Vogelstein, *Science*, 228: 187–190 (1985)). Still other reports have connected hypomethylation of individual genes with tumorigenicity (see e.g., Feinberg and Vogelstein, *Nature*, 301: 89–92 (1983); Jones and Buckley, *Adv. Can. Res.*, 54: 1–12 (1990)). Moreover, it has been suggested that current hypotheses about DNA methylation and cancer suggest that agents that reduce DNA methylation should cause transformation of cells (Jones and Buckley, supra). Thus, the prior art is devoid of any meaningful suggestion of how regulation of DNA methylation may be successfully manipulated to diminish tumorigenicity.

Antisense oligonucleotide technology has allowed for inhibition of expression of a variety of genes. See generally Agrawal, *Trends in Biotech.*, 10: 152 (1992). By binding to the complementary nucleic acid sequence in RNA, antisense oligonucleotides are able to inhibit splicing and translation of RNA. In this way, antisense oligonucleotides are able to inhibit protein expression. Antisense oligonucleotides have also been shown to bind to genomic DNA, forming a triplex, and inhibit transcription. Furthermore, a 17-mer base sequence statistically occurs only once in the human genome, and thus extremely precise targeting of specific sequences is possible with such antisense oligonucleotides.

In 1978 Zamecnik and Stephenson were the first to propose the use of synthetic antisense oligonucleotides for therapeutic purposes. Stephenson and Zamecnik, *Proc. Natl. Acad. Sci. U.S.A.*, 75: 285 (1978); Zamecnik and Stephenson, *Proc. Natl. Acad. Sci. U.S.A.*, 7.5: 280 (1978). They reported that the use of a oligonucleotide 13-mer complementary to the RNA of Rous sarcoma virus inhibited the growth of the virus in cell culture. Since then, numerous other studies have been published manifesting the in vitro efficacy of antisense oligonucleotide inhibition of viral growth, e.g., vesicular stomatitis viruses (Leonetti et al., *Gene*, 72: 323 (1988)), herpes simplex viruses (Smith et al, *Proc. Natl. Acad. Sci. U.S.A.*, 83: 2787 (1986)), and influenza virus (Zerial et al., *Nucleic Acids Res.*, 15: 9909 (1987)).

Antisense oligonucleotides have also been shown to inhibit protein expression from endogenous mammalian genes. For example, Burch and Mahan, *J. Clin. Invest.*, 88: 1190 (1991), disclosed antisense oligonucleotides targeted to murine and human IL-1 receptors that inhibited IL-1-stimulated PGE_2 synthesis in murine and human fibroblasts, respectively; Colige et al., *Biochemistry*, 32: 7 (1993) disclosed antisense oligonucleotides that specifically inhibited expression of a mutated human procollagen gene in transfected mouse 3T3 cells without inhibiting expression of an endogenous gene for the same protein; and Monia et al., *J. Biol. Chem.*, 267: 19954 (1992), disclosed selective inhibition of mutant Ha-ras mRNA expression with phosphorothioate antisense oligonucleotide.

Although antisense approaches have shown promise for a variety of disease-states, there is no clear message about how or whether any genetic target exist that would allow successful use of antisense approaches to affect tumorigenicity. There is, therefore, a need to develop this promising technology in a way that might allow it to be applied in the fight against neoplasia.

SUMMARY OF THE INVENTION

Previous teachings have suggested that agents that inhibit DNA methylation should be capable of transforming cells (see e.g., Jones & Buckley, *Adv. in Cancer Res.*, 54: 1–23 (1990)).

The present invention provides antisense oligonucleotides that surprisingly demonstrate tumorigenicitiy-inhibiting activity. The inventive oligonucleotides inhibit tumorigenisis by inhibiting expression of the gene encoding DNA methyl transferase. These oligonucleotides are complementary to mRNA or double-stranded DNA that encodes mammalian DNA methyl transferase. The present invention further provides useful compounds, compositions and methods for preventing the expression of the DNA methyl transferase gene. A still further object of the present invention is to provide compounds, compositions and methods for the treatment of and inhibition of tumorigenicity.

Accordingly, this disclosure presents antisense oligonucleotides that have been constructed and are targeted to bind to nucleic acid sequences encoding DNA MeTase, thereby blocking production of the expression product. Also presented are methods for inhibiting DNA MeTase expression and tumorigenesis.

The invention is useful in curing experimental mice of tumors. More specifically, the invention is useful in curing

nude mice of human tumors, and, in particular, human small lung cell carcinoma. The invention may thus be used to avoid sacrificing an animal at the end of an experiment.

The present invention provides methods for inhibiting tumorigenesis by expressing an antisense message to the DNA MeTase in a cell line, and specifically in mouse and human cancer cell lines. Expression of an antisense DNA MeTase leads to: (i) a general reduction in the methylation content of the genome; (ii) demethylation of regions aberrantly methylated in a cell line such as the adrenal specific 21-hydroxylase gene as well as tumor suppressor loci; (iii) morphological changes indicative of inhibition of the transformed phenotype; (iv) inhibition of tumorigenesis in vitro as well as a loss of angiogenic function; and (vi) to the ability to undergo an apoptotic death program under appropriate conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a physical map of the plasmids pZEM and pZαM. The metallothionine (MT) promoter (shaded box), the human growth hormone 3' region (HGH) (open bar), and the MeTase cDNA sequences (hatched) are indicated.

FIG. 2 is a graph showing the state of methylation of total genomic DNA and specific genes in $Y1_p$ZαM transfectants. The spots on TLC plates corresponding to C and 5-methyl C were scraped and counted in a liquid β scintillation counter. The values represent the means±SEM.

FIG. 3 is a graph indicating anchorage independent growth assay of: Y1 pZEM (clones 4 and 7) and Y1 pZαM transfectants (clones 4, 7 and 9).

FIG. 4 is a graph indicating a loss of antisense expression in tumors derived from Y1 pZαM transfectants.

FIG. 5*a* is a graph showing survival and apoptosis of Y1 pZαM cells as determined by a density restricted growth assay.

FIG. 5*b* is a graph showing survival and apoptosis of Y1 pZαM cells in serum deprived medium.

FIG. 6 is a graph showing the percentage of CpG methylation in NCI H446 cells expressing antisense to DNA MeTase and in cells expressing a DNA MeTase sense control oligonucleotide.

FIG. 7 shown the ability of NCI H446 cells treated with antisense and control oligonucleotides to grow in an anchorage independent fashion in soft agar.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention provides antisense oligonucleotides that surprisingly inhibit tumorigenicity. These oligonucleotides are complementary to mRNA or double-stranded DNA that express mammalian, and, in particular, human or mouse, DNA methyl transferase and unexpectedly display tumorigenicity-inhibiting activity. One preferred antisense oligonucleotide of the present invention is 5'-CATCTGCCATTCCCACTCTA-3' (SEQ ID NO 1), having either phosphodiester or phosphorothioate linkages. Other suitable antisense oligonucleotides include the phosphorothioate: 5'-TTGGCATCTGCCATTCCCACTCTA-3' (SEQ ID NO 2).

Modified oligonucleotides having in vivo activity against tumorigenicity are referred to herein as anti-tumorigenicity or tumorigenicity-inhibiting modified oligonucleotides. The invention provides tumorigenicity-inhibiting modified oligonucleotides that have efficacy in inhibiting expression of DNA methyl transferase. Modified oligonucleotides according to the invention have specific preferred characteristics that are discussed in greater detail for each preferred embodiment below. In addition to these characteristics, modified oligonucleotides according to the invention may optionally have additional ribonucleotide, 2'-substituted ribonucleotide, and/or deoxyribonucleotide monomers, any of which are connected together via 5' to 3' linkages which may include any of the internucleotide linkages known in the art. Preferably, such modified oligonucleotides may optionally contain phosphodiester, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate and/or sulfone internucleotide linkages. Those skilled in the art will recognize that the synthesis of oligonucleotides containing any of these internucleotide linkages is well known to those skilled in the art, as is illustrated by articles by Uhlmann and-Peyman, *Chemical Reviews*, 90: 543–584 (1990) and Schneider and Banner, *Tetrahedron Lett.*, 21: 335 (1990). Preferably, modified oligonucleotides according to the invention should contain from about 6 to about 100 monomers in total and most preferably from about 12 to about 50 total monomers. Such modified oligonucleotides may also optionally contain modified nucleic acid bases and/or sugars, as well as added substituents, such as diamines, cholesteryl or other lipophilic groups.

Various preferred embodiments of modified oligonucleotides according to the invention are discussed below. Although these embodiments all have a nucleotide sequence from the same region of the DNA MeTase gene, those skilled in the art will recognize that the tumorigenicity-inhibiting efficacy of oligonucleotides having nucleotide sequences complementary to other essential nucleic acid sequences of DNA MeTase can also be enhanced by incorporating into such oligonucleotides the structural features of preferred embodiments of modified oligonucleotides according to the invention.

For purposes of the invention, complementary means having a sequence that hybridizes to the essential nucleic acid sequence under physiological conditions. An essential nucleic acid sequence of the DNA MeTase gene means a nucleic acid sequence that is required for expressing DNA MeTase. For example, such oligonucleotides can have other sequences from the DNA MeTase gene. Indeed, any sequence from the DNA MeTase gene (the 5'-region as disclosed by Rouleau et al, *J. Biol. Chem.*, 267: 7368–7377 (1992) or Yen et al., *Nucl. Acids Res.*, 9: 2287–2291 (1992) should serve as the basis for modified oligonucleotides according to the invention. As a practical matter, the structural features of preferred embodiments of modified oligonucleotides according to the invention should enhance the tumorigenicity-inhibiting activity of any antisense oligonucleotide having a nucleotide sequence that hybridizes in a cell with any essential nucleic acid sequence of the DNA MeTase gene.

Each preferred embodiment of modified oligonucleotides according to the invention is separately discussed in greater detail below.

In a first preferred embodiment, tumorigenicity-inhibiting modified oligonucleotides according to the invention are in the form of a mixed backbone or chimeric oligonucleotide having one or more regions of nucleotides connected by phosphorothioate or phosphorodithioate internucleotide linkages ("phosphorothioate or phosphorodithioate region") as well as one or more regions of nucleotides connected by

alkylphosphonate internucleotide linkages ("alkylphosphonate region"). In this embodiment, at least one alkylphosphonate region preferably includes nucleotides at or near the 5' end and/or the 3' end of the oligonucleotide. For purposes of the invention, at or near the 5' or the 3' end of the oligonucleotide means involving at least one nucleotide within about 5 nucleotides from the 5' or 3' end of the oligonucleotide. Preferably, the alkylphosphonate region comprises from about 2 to about 10 contiguous nucleotides connected by alkylphosphonate linkages. Preferably, the phosphorothioate or phosphorodithioate region comprises at least 3, and up to about 100 contiguous nucleotides connected by phosphorothioate or phosphorodithioate linkages. Many embodiments of oligonucleotides having this type of backbone structure are taught in U.S. Pat. Nos. 5,149,797 and 5,220,007, the teachings of which are hereby incorporated by reference.

Modified oligonucleotides having tumorigenicity-inhibiting activity according to this embodiment of the invention are synthesized by solid phase methods, alternating H-phosphonate chemistry and sulfur oxidation for phosphorothioate regions, and alkylphosphonamidate chemistry for alkylphosphonate regions. A preferred H-phosphonate approach is taught by Agrawal et al., U.S. Pat. No. 5,149,798, the teachings of which are hereby incorporated by reference. Alkylphosphonamidite chemistry is well known in the art, as illustrated by Agrawal and Goodchild, *Tetrahedron Lett.*, 28: 3539–3542 (1987). Synthesis of phosphorodithioate-containing oligonucleotides is also well known in the art, as illustrated by U.S. Pat. No. 5,151,510, the teachings of which are hereby incorporated by reference (See also, e.g., Marshall and Caruthers, *Science*, 259: 1564–1570 (1993) and references cited therein).

In a second preferred embodiment, modified oligonucleotides having tumorigenicity-inhibiting activity according to the invention are in the form of a mixed backbone of chimeric oligonucleotide having one or more region of nucleotides connected by phosphorothioate or phosphorodithioate internucleotide linkages ("phosphorothioate or phosphorodithioate region"), as well as one or more region of nucleotides connected by alkylphosphonothioate or arylphosphonothioate internucleotide linkages ("alkylphosphonothioate region"). In this embodiment, at least one alkylphosphonothioate region preferably includes nucleotides at or near the 5' end and/or the 3' end of the oligonucleotide. Preferably, the alkylphosphonothioate region comprises from about 2 to about 10 contiguous nucleotides connected by alkylphosphonothioate linkages. Preferably, the phosphorothioate or phosphorodithioate region comprises at least 3, and up to about 100 contiguous nucleotides connected by phosphorothioate or phosphorodithioate linkages.

Tumorigenicity-inhibiting modified oligonucleotides according to this embodiment of the invention are synthesized by solid phase methods, alternating chemistries for each region to be synthesized. Phosphorothioate or phosphorodithioate regions are synthesized as described for the first embodiment. Alkylphosphonothioate regions are synthesized by coupling together two or more nucleosides via alkylphosphite linkages, then oxidatively thiolating the alkylphosphite linkages to produce alkylphosphonothioate linkages (see e.g., Agrawal et al., *Nucl. Acids Res.*, 20: 2729–2735 (1993).

In a third preferred embodiment, tumorigenicity-inhibiting modified oligonucleotides according to the invention are in the form of a hybrid oligonucleotide having regions of deoxyribonucleotides ("deoxyribonucleotide regions") and regions of ribonucleotides or 2'-substituted ribonucleotides ("ribonucleotide regions"). Preferably, from about one to about all of the internucleotide linkages are phosphorothioate or phosphorodithioate linkages. Preferred 2'-substituted ribonucleotides are halo, amino, alkyl, aryl or lower alkyl (1–6 carbon atoms) substituted ribonucleotides, especially 2'-OMe-ribonucleotides. Preferably, at least some of the ribonucleotide regions include nucleotides present at or near the 5' end and/or the 3' end of the oligonucleotide. Most preferably, the ribonucleotide regions each comprise from about 2 and preferably from about 4 to about 100 contiguous ribonucleotides and/or 2'-substitute oligonucleotides. The deoxyribonucleotide regions are optional, and when present may contain from about 1 to about 100 contiguous deoxyribonucleotides. Tumorigenicity-inhibiting modified oligonucleotides according to this embodiment of the invention are typically synthesized by solid phase methods, preferably by the phosphoramidite approach, using deoxynucleotide phosphoramidites for deoxyribonucleotide regions, and ribonucleotide or 2'-substituted ribonucleotide phosphoramidite for ribonucleotide regions.

In a fourth preferred embodiment, tumorigenicity-inhibiting modified oligonucleotides according to the invention are in the form of an oligonucleotide having at its 5' and/or 3' end a cap structure that confers exonuclease resistance to the oligonucleotide. Such modified oligonucleotides preferably also have from 1 to about all modified (non-phosphodiester) internucleotide linkages. Preferred cap structures include lower alkyl (C_1–C_{12}) or alcohol groups. Preferred modified internucleotide linkages include phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate, sulfone, phosphorothioate and phosphorodithioate linkages. Tumorigenicity-inhibiting modified oligonucleotides according to this embodiment of the invention are synthesized according to procedures well known in the art (see e.g., Uhlmann and Peyman, *Chemical Reviews,* 90: 43–584 (1990) ; Schneider and Banner, *Tetrahedron Lett.*, 31: 335 (1990)). For oligonucleotides having cap structures at the 3' end, the cap structure is reversibly attached to the solid support and is then coupled to the first nucleotide monomer in the synthesis scheme. For oligonucleotides having cap structures at the 5' end, the cap structure is coupled to the end of the oligonucleotide after addition of the last nucleotide monomer in the synthesis scheme.

In a fifth embodiment, tumorigenicity-inhibiting modified oligonucleotides are self-stabilized by having a self-complementary region that hybridizes intramolecularly with the oligonucleotide to form an exonuclease resistant hairpin-like structure (see e.g., Agrawal et al., *Nucleic Acids Res.* 20: 2729–2735 (1993). Modified oligonucleotides according to this embodiment of the invention are generally characterized by having two regions: a DNA MeTase hybridizing region and a self-complementary region. The DNA MeTase hybridizing region has a nucleotide sequence that is complementary to an essential nucleic acid sequence of DNA MeTase. Preferably, this region has from about 6 to about 100 nucleotides. In this embodiment, the oligonucleotide is stabilized, i.e., rendered resistant to exonucleolytic degradation by base-pairing between the target hybridizing region and the self-complementary region and/or by base-pairing between complementary sequences within the self-complementary region. When the oligonucleotide encounters a DNA MeTase nucleic acid molecule having a complementary nucleic acid sequence, base-pairing between the DNA MeTase hybridizing region and the self-complementary

region of the oligonucleotide is disrupted and replaced by base-pairing between the DNA MeTase hybridizing region of the oligonucleotide and the complementary nucleic acid sequence of the nucleic acid molecule. This disruption and replacement of base-pairing takes place because the intermolecular base-paired structure formed by the hybrid between the target nucleic acid sequence and the target hybridizing region is more thermodynamically stable than the intramolecular base-paired structure formed by the self-complementary oligonucleotide.

A second form of an oligonucleotide according to this embodiment of the invention operates in a similar way as the first form, but forms a different structure upon self-complementary base-pairing. This alternative form forms a hammer-like structure. In this form, the self-complementary region contains oligonucleotide sequences that can base pair with other oligonucleotide sequences within the self-complementary region. The self-complementary region may also contain oligonucleotide sequences that are complementary to the tumorigenicity hybridizing region.

The second significant region of self-stabilized oligonucleotides according to the invention is the self-complementary region. The self-complementary region contains oligonucleotide sequences that are complementary to other oligonucleotide sequences within the oligonucleotide. These other oligonucleotide sequences may be within the DNA MeTase hybridizing region or within the self-complementary region, or they may span both regions. The complementary sequences form base pairs, resulting in the formation of a hairpin structure or a hammer-like structure. Either the hairpin structure or the hammer-like structure will presumably have loops of 4 or more nucleotides resulting from non-base-paired nucleotides. The number of base-pairs to be formed by intramolecular hybridization involving the self-complementary region may vary, but should be adequate to maintain a double-stranded structure so that the 3' end is not accessible to endonucleases. Generally, about 4 or more base-pairs will be necessary to maintain such a double-stranded structure. In a preferred embodiment, there are about 10 intramolecular base-pairs formed in the self-stabilized oligonucleotide, with the 10 base pairs being consecutive and involving the 3'-most nucleotides. Of course, the intramolecular base-pairing can be so extensive as to involve every nucleotide of the oligonucleotide. Preferably, this will involve a self-complementary region of about 50 nucleotides or less.

Oligonucleotides according to this embodiment may have from 1 to about all modified internucleotide linkages, as described for the fourth embodiment. Preferably, at least either the DNA MeTase hybridizing region or the self-complementary region, and most preferably both, will contain from about 2 to about all nucleotides being coupled by phosphorothioate and/or phosphorodithioate linkages.

Those skilled in the art will recognize that the features of the various preferred embodiments described above can be combined to produce additional embodiments that may have even greater tumorigenicity-inhibiting activity. Thus, the invention contemplates modified tumorigenicity-inhibiting oligonucleotides having every possible combination of chimeric features, hybrid features, cap structures and self-stabilizing character, all as described herein. Such oligonucleotides are useful as therapeutic agents for inhibition of tumor growth. For such treatment, oligonucleotides may be administered intraperitoneally, intranasally, orally or anally. Preferably, such oligonucleotides will be administered at a concentration of from about 1 to about 50 mg/kg body weight.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in nature.

EXAMPLE 1

Expression of antisense to the DNA Methyltransferase gene in Y1 cells results in limited DNA demethylation

Cell Culture and DNA Mediated Gene Transfer

To directly inhibit DNA methylation in Y1 cells, either the DNA MeTase antisense expression construct pZαM or a pZEM control vector, Szyf, et al., *J. Biol. Chem.*, 267: 12831–12836 (1992)) was introduced into Y1 adrenocortical carcinoma cells by DNA-mediated gene transfer as follows.

Y1 cells were maintained as monolayers in F-10 medium which was supplemented with 7.25% heat inactivated horse serum and 2.5% heat inactivated fetal calf serum (Immunocorp. Montreal) (Yasumura, et al., *Cancer Res.*, 26: 529–535 (1988)). All other media and reagents for cell culture were obtained from GIBCO-BRL. Y1 cells (1×10^6) were plated on a 150 mm dish (Nunc) 15 hours before transfection. The pZαM expression vector (10 μg) was cointroduced into Y1 cells with 1 μg of pUCSVneo as a selectable marker by DNA mediated gene transfer using the calcium phosphate protocol (Ausubel, et al., 1988, Current Protocols in Molecular Biology, Wiley and Sons, New York). Selection was initiated 48 hours after transfection by adding 0.25 mg/ml G418 (GIBCO-BRL) to the medium. For both constructs, G418 resistant cells were isolated and then cloned in selective medium. For analysis of growth in soft agar, 1×10^3 cells were seeded in triplicate onto 30 mm dishes (Falcon) with 4 ml of F-10 medium containing 7.5% horse serum, 2.5% FCS, 0.25 mg/ml G418 (for transfectants) and 0.33% agar solution at 37° C. (Freedman and Shin, *Cell*, 3: 355–359 (1974)). Cells were fed with 2 ml of medium plus G418 every two days. Growth was scored as colonies containing $>10^2$ cells, 21 days after plating.

EXAMPLE 2

DNA and RNA Analyses

Preparation of genomic DNA and total cellular RNA, labelling (using the random primer labelling kit from Boehringer Mannheim), blotting RNA on to Hybond-N+(Amersham), and all other standard molecular biology manipulations were performed according to Ausubel et al., 1988, Current Protocols in Molecular Biology. Wiley and Sons, New York. MspI and HpaII restriction enzymes (Boehringer Mannheim) were added to DNA at a concentration of 2.5 units/ug for 8 h at 37° C. Radionucleotides (3000 mCi/mmol) were purchased from Amersham.

To confirm that the transfectants bear the introduced construct, DNA was prepared from the transfectants and subjected to digestion by either MspI or HpaII, Southern blot analysis and hydridization with a ^{32}P labelled 0.6 kb DNA MeTase cDNA fragment. The results demonstrated that the three pZαM transfectants contained significant levels of the DNA MeTase cDNA sequence while the control transfectants were clean.

To test whether the pZαM constructs is expressed in the transfectants and whether the metallothionein promoter is functional in these cells, the transfectants were cultured with 50 μM of ZnSO4, RNA prepared at different time points and

subsequently subjected to Northern blot analysis and hybdridization with the ^{32}P labelled MET 0.6 probe. Transfectants 7 and 9 express substantial amounts of the MET 0.6 cDNA (~1.3 kb chimeric mRNA) even before induction with ZnSO4.

EXAMPLE 3

Demethylation of specific genes in Y1 pZαM transfectants

To verify that expression of pZαM results in demethylation and to determine whether specific genes were demethylated, HpaII/MspI restriction enzyme analysis was employed followed by Southern blotting and hybridization with specific gene probes. HpaII cleaves the sequence CCGG, a subset of the CpG dinucleotide sequences, only when the site is unmethylated while MspI will cleave the same sequence irrespective of its state of methylation. The pattern of HpaII cleavage of specific genes in cells expressing pZαM was compared with that of the parental Y1 or cells harboring only the vector to determine whether the genes are demethylated in the antisense transfectants. The state of methylation of the steroid 21-hydroxylase gene C21 was analyzed first. (Szyf et al., *Proc. Natl. Acad. Sci. USA,* 86: 6853–6857 (1989); Szyf, et al., *Mol. Endocrin.,* 4: 1144–1152 (1990)). This gene is specifically expressed and hypomethylated in the adrenal cortex but is inactivated and hypermethylated in Y1 cells (Szyf et al., *Proc. Natl. Acad. Sci. USA,* 86: 6853–6857 (1989)); Szyf, et al., *Mol. Endocrin.,* 4: 1144–1152 (1990)). DNA prepared from Y1, pZαM (Bernards, et al., *Proc. Natl. Acad. Sci. USA,* 86: 6474–6478 (1989)); Collins et al., *J. Exp. Med.,* 76: 1043–1091 (1992)) and pZEM (Bernards, et al., *Proc. Natl. Acad. Sci. USA,* 86: 6474–6478 (1989)) transfectants was subjected to either MspI or HpaII digestion, Southern blot analysis and hybridization with a 0.36 kb Xba-BamHI fragment containing the enhancer and promoter regions of the C21 gene (see Szyf et al., *Proc. Natl. Acad. Sci. USA,* 86: 6853–6857 (1989); Szyf, et al., *Mol. Endocrin.,* 4: 1144–1152 (1990) for physical map of the probe). This probe detects 0.36 kb and 0.16 kb HpaII fragments when the promoter region is fully demethylated (Szyf et al., *Proc. Natl. Acad. Sci. USA,* 86: 6853–6857 (1989); Szyf, et al., *Mol. Endocrin.,* 4: 1144–1152 (1990)).

The promoter and enhancer region is heavily methylated in Y1 cells and the pZEM transfectants. In contrast, the Y1 pZαM transfectants bear a partially demethylated C21 5' region as indicated by the relative diminution of the 3.8 and 2 kb fragments and the appearance of the fully demethylated faint bands at 0.36 kb as well as the fact that HpaII cleavage yields partial fragments at 0.56 and ~1 kb indicating partial hypomethylation of sites upstream and downstream to the enhancer region.

To determine whether hypomethylation was limited to the enhancer region or spread throughout the C21 gene locus, similar HpaII digestion and Southern blot transfer were performed on different preparations of DNA extracted from Y1 cells, a control pZEM (Bernards, et al., *Proc. Natl. Acad. Sci. USA,* 86: 6474–6478 (1989)) transfectant, and three pZαM antisense transfectants. The filter was hibridized with a 3.8 kb BamHI fragment containing the body of the C21 gene and 3' sequences (Szyf et al., *Proc. Natl. Acad. Sci. USA,* 86: 6853–6857 (1989); Szyf, et al., *Mol. Endocrin.,* 4: 1144–1152 (1990) for physical map). Full demethylation of this region yields a doublet at ~1 kb, a 0.8 kb fragment and a 0.4 kb fragment as well as a number of low molecular weight fragments at 0.1–0.2 kb. The C21 locus is heavily methylated in Y1 cells as well as the control transfectant as indicated by the high molecular weight fragments above 23 kb. Only a faint band is present in the expected 1 kb molecular weight range as well as a partial at 1.9 kb as well as the appearance of new partial fragments in the lower molecular weight range between 1 and 0.4 kb indicating partial hypomethylation at a large number of HpaII sites contained in the 3' region of the C21 gene (Szyf et al., *Proc. Natl. Acad. Sci. USA,* 86: 6853–6857 (1989); Szyf, et al., *Mol. Endocrin.,* 4: 1144–1152 (1990)). The pattern of demethylation, indicated by the large number of partial HpaII fragments, is compatible with a general partial hypomethylation rather than a specific loss of methylation in a distinct region of the C21 gene.

To determine whether demethylation is limited to genes that are potentially expressible in Y1 cells such as the adrenal cortex-specific C21 gene (Szyf, et al., *Mol. Endocrin.,* 4: 1144–1152 (1990)) or if the demethylation is widely spread in the genome, other genes such as the muscle specific MyoD gene as well as the hippocampus specific 5HT1A receptor gene were analyzed; both genes were hypomethylated.

Another class of genes that might have undergone a specific hypomethylation includes the tumor suppressor genes. The state of methylation of two genes from this class was determined, p53 and retinoblastoma (RB) which are both tumor suppressor genes involved in cell cycle regulation. Loss of either one of these gene products has been shown to lead to deregulation of the cell cycle and neoplasia (Bernards, et al., *Proc. Natl. Acad. Sci. USA,* 86: 6474–6478 (1989); Donehoweer, et al., *Nature,* 356: 215–221 (1992)).

Generation of p53 and retinoblastoma RB) probes by PCR

Oligoprimers for the 5' region of the mouse p53 gene were selected from the published genomic sequence (Accession number: XO1235) (Zakut-Houri, et al., *Nature* 306: 594–597 (1983)) using the Primer selecting program (PC Gene). The 5' primer corresponding to bases 154–172: 5'TCC GAA TCG GTT TCC ACCC 3' (SEQ ID NO 3) and the 3' primer corresponding to bases 472–492 5' GGA GGA TGA GGG CCT GAA TGC 3' (SEQ ID NO 4) were added to an amplification recation mixture containing 100 μg of mouse DNA (from C2C12 cells) using the incubation conditions recommended by the manufacturer (Amersham Hot tub) (1.5 mM $MgCl_2$) and the DNA was amplified for 40 cycles of 2 minutes at 95° C., 2 minutes at 55° C. and 0.5 minutes at 72° C. The reaction products were separated on a low-melt agarose gel (BRL) and the band corresponding to the expected size was excised and extracted according to standard protocols (Ausubel, et al., 1988, Current Protocols in Molecular Biology. Wiley and Sons, New York).

Since the genomic sequence of the mouse RB gene was unavailable through Genbank we reverse transcribed the retinoblastoma mRNA from 0.5 μg of total mouse RNA (from C2C12 cells) using random oligonucleotide primers (Boehringer) with Superscript reverse transcriptase (BRL) under conditions recommended by the manufacturer. The RB sequence was amplified from the reverse transcribed cDNA using oligonucleotides corresponding to bases 2–628 of the published cDNA (Bernards et al., *Proc. Natl. Acad. Sci. USA,* 86: 6474–6478 (1989)). The oligoprimers used were 5' GGA CTG GGG TGA GGA CGG 3' (1–18) (SEQ ID NO 5) and 5' TTT CAG TAG ATA ACG CAC TGC TGG 3' (620–610) (SEQ ID NO 6). The amplification conditions were as described above.

Using a probe to a 300 bp sequence from the 5' region of the mouse RB cDNA, the level of methylation of this gene was determined in Y1 cells transfected with a control vector as well as the pZαM transfectants. Cleavage of this region with HpaII yields 0.6 kb and 0.1 kb fragments. The RB locus is heavily methylated in the control cells as indicated by hybridization of the probe to high molecular weight fragments. This locus is partially hypomethylated in the pZαM transfectants as indicated by the relative diminution in the intensity of the high molecular weight markers and the partial presence of the 0.6 and 0.15 kb fragments.

EXAMPLE 4

Nearest neighbor analysis

To determine whether expression of antisense RNA to the DNA MeTase gene leads to a general reduction in the level of methylation of the genome, "nearest neighbor" analysis using [α-^{32}P]-dGTP was conducted as described by Razin et al., 1985, in Razin, A., and G. L. Cantoni. (Ed), Biochemistry and Biology of DNA methylation, Allan R. Liss, Inc. New York. This assay enables a determination of the percentage of methylated cytosines residing in the dinucleotide sequence CpG. Transfectants and control DNAs were nicked with DNAaseI, nick translated with a single nucleotide [α-^{32}P]-dGTP using DNA polymerase I and the labelled DNA was digested to 3' mononucleotide phosphates with micrococal nuclease which cleaves DNA 3' to the introduced α-^{32}P. The [α-^{32}P] labelled 5α neighbors of dGMP were separated by chromatography on a TLC plate, the resulting spots for dCMP and dC^{met}MP were scraped and counted by liquid scintillation. The results of a triplicate experiment presented in FIG. 2*a* (sample autoradiogram) and b (graphic representation) suggest that a limited but significant reduction in the total level of DNA methylation (12% for transfectant number 4 and 22% for 7) occurred in transfectants expressing the pZαM construct when compared to the control line pZEM.

"Nearest Neighbor" analysis was performed as follows: 2 μg of DNA were incubated at 37° C. for 15 minutes with 0.1 unit of DNAase, 2.5 μl of ^{32}P-α-dGTP (3000 Ci/mmol from Amersham) and 2 units of Kornberg DNA polymerase (Boehringer) were then added and the reaction was incubated for an additional 25 minutes at 30° C. 50 μl of water were added and the nonincorporated nucleotides were removed by spinning through a microcon column (Amicon) at maximum speed for 30 seconds. The labelled DNA (20 μl) was digested with 70 μg of micrococal nuclease (Pharmacia) in the manufacturer's recommended buffer for 10 hours at 37° C. Equal amounts of radioactivity were loaded on TLC phosphocellulose plates (Merck) and the 3' mononucleotides were separated by chromatography in one dimension (isobutyric acid: H_2O: NH_4OH in the ratio 66:33:1). The chromatograms were exposed to XAR film (Eastman-Kodak) and the spots corresponding to cytosine and 5-methylcytosine were scraped and counted in a β-scintillation counter.

EXAMPLE 5

In Vitro Tumorigenicity Assays

While control Y1 and Y1 pZEM cells exhibit limited contact inhibition and form multilayer foci, Y1 pZαM transfectants exhibit a more rounded and distinct morphology and grow exclusively in monolayers.

To determine whether the expression of antisense to the DNA MeTase results in reversal of the tumorigenic potential, the ability of the transfectants to grow in an anchorage independent fashion was determined. This assay is considered an indicator of tumorigenicity (Freedman and Shin, *Cell* 3: 355–359 (1974)). The Y1 pZαM transfectants demonstrate an almost complete loss of ability to form colonies in soft agar, moreover the colonies that do form contain only a few cells as demonstrated (FIG. 3B). Growth on soft agar was quantified by visual examination and presented graphically in FIG. **3**. These experiments demonstrate that inhibition of DNA methylation by expression of an antisense message to the DNA MeTase leads to loss of tumorigenicity in vitro.

EXAMPLE 6

In Vivo Tumorigenicity Assays

Syngenic LAF-1 mice (6–8 week old males) were injected subcutaneously (in the flank area) with 10^6 cells of each of the Y1 pZαM, Y1 and Y1 pZEM transfectants. Mice were monitored for the presence of tumors by daily palpitation. Mice bearing tumors of greater than 1 cm in diameter were sacrificed by asphyxiation with CO_2, tumors were removed by dissection and homogenized in guanidium isothiocyanate. Mice that were tumor free were kept for ninety days and then sacrificed. RNA was prepared from the tumors by $CsCl_2$ (Ausubel, et al., 1988, Current Protocols in Molecular Biology, Wiley and Sons, New York).

The presence of tumors was determined by palpitation. While all the animals injected with Y1 cells formed tumors two to three weeks post injection, the rate of tumor formation in the animals injected with the pZαM transfectants was significantly lower. The results are shown below in Table 1.

TABLE 1

Cell line injected	Tumors	Neovascularization
Y1	6/6	+++
pZEM 4	5/5	+++
pZαM 4	1/6	—
pZαM 7	2/6	—
pZαM 9	2/6	—

EXAMPLE 6A

In Vivo Inhibition of Tumorigenicity of Human Small Lung Carcinoma Cells in a Nude Mouse System

To determine whether inhibition of DNA MeTase by expression of an antisense message results in inhibition of cellular transformation of human carcinomas, a 330 bp sequence containing the translation initiation site (+155–+481) was amplified using the published human DNA MeTase cDNA sequence using the amplification protocol described above in Example 3 (antisense primer was: 5' GCA AAC AGA ATA AAG AAT C 3' (SEQ ID NO 7), the sense primer was: 5' GTA TGG TGG TTT GCC TGG T 3' (SEQ ID NO 8)). The 330 bp sequence was subcloned in the antisense orientation into the expression vector pZEM as described above for the mouse antisense. A human small lung carcinoma cell line NCI H446 was cotransfected with either an antisense DNA MeTase expression vector or a control sense expression vector and a plasmid conferring resistance to hygromycin using transfection protocols as

described above. Hygromycin resistant colonies were selected and the presence of the transfected antisense was verified by digestion with EcoRI, Southern blot transfer and hybridization with a 0.4 kb human DNA MeTase cDNA probe. Demethylation of genomic DNA of cells expressing the antisense was verified by nearest neighbor analysis (FIG. 6) as described above and by hybridization with specific gene probes. The gene encoding the IGF-1 growth factor was demethylated in antisense transfectants but not sense controls.

To determine whether the expression of antisense to DNA MeTase results in reversal of the tumorigenic potential, the ability of the transfectants to grow in an anchorage independent fashion was analyzed. The antisense transfectants lost their ability to form colonies in soft agar indicating loss of tumorigenicity in vitro.

Tumor growth in nude mice was evaluated as follows:

4 groups of mice were injected with 10^6 NCI H446 cells transfected with the pZαM 5' human MeTase (0.4 kb) antisense expression plasmid and the hygromycin resistance plasmid.

1 group of mice was injected with 10^6 NCI H446 cells transfected with the pZαM 5' human MeTase (0.4 kb) sense expression plasmid and the hygromycin resistance plasmid.

1 group of mice was injected with 10^6 NCI H446 cells bearing the hygromycin resistance plasmid.

1 group of mice was injected with 10^6 NCI H446 lung cell line.

The mice were followed for in excess of 12 weeks. The results are shown in Table II. These results demonstrate that expression of antisense to the DNA MeTase inhibited turigenesis in vivo.

TABLE II

TUMOR DEVELOPMENT IN NUDE MICE

Transfectant clones	Number of mice injected	Number of mice developing tumors	Latency period of mice developing tumors
pZαM[1] #3	3	0	>12 weeks
pZαM #3	3	0	>12 weeks
pZαM #3	2	0	>12 weeks
pZαM #3	3	0	>12 weeks
pZM[2] #5	3	2	5 weeks
Hyg only[3]	3	3	5 weeks
Tumor only[4]	3	3	3 weeks

[1]NCI H446 cells transfected with the pZαM 5' human MeTase (0.4 kb) antisense expression plasmid and the hygromycin resistance plasmid
[2]NCI H446 cells transfected with the pZαM 5' human MeTase (0.4 kb) sense expression plasmid and the hygromycin resistance plasmid
[3]NCI H446 cells bearing the hygromycin resistance plasmid
[4]NCI H446 lung cell line

Neovascularization

Many lines of evidence suggest that angiogenic potential and metastatic potential of cell lines are directly related (Liotta, et al., *Cell*, 64: 327–336 (1991)). The tumors that do arise from the pZαM transfectants exhibit very limited neovascularization while tumors that formed in the animals that were injected with Y1 cells or control transfectants were highly vascularized.

RNA from a tumor arising from the Y1pZαM transfectant was isolated and the level of expression of the 0.6 kb antisense message was compared with that observed for the transfectant line in vitro. The isolated RNAs were subjected to Northern blot analysis and hybridization with a ^{32}P labelled MET 0.6 fragment. The filter was stripped of its radioactivity and was rehybridized with a ^{32}P labelled oligonucleotide probe for 18S rRNA as previously described (Szyf et al., *Mo. Endocrinol.*, 4: 1144–1152 (1990)). The autoradiograms were scanned and the level of expression of MET 0.6 was determined relative to the signal obtained with the 18S probe. The expression of the antisense message is significantly reduced in the tumors. Thus, it appears that expression of an antisense message to the DNA MeTase is incompatible with tumorigenesis. Apparently, the small number of tumors that did form in animals injected with the pZαM transfectants were derived from revertants that lost expression of the antisense to the DNA MeTase under the selective pressure in vivo.

EXAMPLE 7

Relationship of Serum Deprivation and Expression of pZαM in Y1 Cells to Apoptotic Death Program

Tumor cells exhibit limited dependence on serum and are usually capable of serum independent growth (Barns and Sato, *Cell*, 22: 649–655 (1980)). Factors present in the serum are essential for the survival of many nontumorigenic cells. Several lines of evidence have recently suggested that the enhanced survivability of tumorigenic cells is associated with inhibition of programmed cell death. For example, the oncogene bcl-2 is not a stimulator of cell proliferation but rather causes inhibition of apoptosis (Strasser, et al., *Nature*, 348: 331–333 (1990)). The tumor suppressor p53 can induce apoptosis in a human colon tumor derived line (Shaw, et al., *Proc. Natl. Acad. Sci.*, 89: 4495–4499 (1992)) and certain chemotherapeutic agents have been shown to incude apoptosis in cancer cells (Collins et al, *J. Exp. Med.*, 176: 1043–1091 (1992)).

Observation of the pZαM transfectants indicated that they exhibited enhanced dependence on serum and limited survivability under serum deprived conditions. The effects of serum starvation were studied on pZαM transfectants. pZαM transfectants and control Y1 pZEM transfectants (3×10^5 per well) were plated in low serum medium (1% horse serum) in six well plates, harvested every 24 hours and tested for viability by trypan blue staining (FIG. 6B). While the control cells exhibited almost 100% viability up to 72 hours after transfer into serum deprived medium, the Y1pZαM cells showed up to 75% loss of viability at 48 hours (FIG. 6B).

Y1 pZαM cells were plated in starvation medium (1% horse serum) and harvested at 24 hour intervals. Total cellular DNA was isolated from the cells and was subjected to electrophoresis on a 1.5% agarose gel followed by transfer to nylon membrane and hybridization with random labeled Y1 genomic DNA. After 48 hours in serum starved conditions, pZαM transfectants exhibit the characteristic 180 bp internucleosomal DNA ladder while the control pZEM transfectants show no apoptosis at this time point.

Y1 pZαM cells were serum starved for 24 hours (2% horse serum), harvested and analyzed by electron microscopy as follows. Cells were fixed in glutaraldehyde (2.5%) in cacodylate buffer (0.1M) for one hour and further fixed in 1% osmium tetroxide. The samples were dehydrated in ascending alcohol concentrations and propylene oxide followed by embedding in Epon. Semi-thin sections (1 μM) were cut from blocks with an ultramicrotome, counterstained with uranil acetate and lead citrate. Samples were analyzed using a Philips 410 electron microscope (Maysinger, et al., *Neurochem. Intl.*, 23: 123–129 (1993)).

Electron microscopy of control Y1 pZEM and Y1 pZαM transfectants at various magnifications revealed that control cells have a fine uniform nuclear membrane whereas the pZαM cells exhibit the cardinal markers of apoptosis (Wyllie, et al., *Histochem. J.*, 13: 681-692 (1981)) condensation of chromatin and its margination at the nuclear periphery, chromatin condensation, nuclear fragmentation, formation of apoptotic bodies and cellular fragmentation. This set of experiments suggests that one possible mechanism through which demethylation can inhibit tumorigenesis is by eliminating the inhibition of programmed cell death.

EXAMPLE 8

In this experiment, human small lung carcinoma cells (NCI H446) were treated with 5 μl lipofectin reagent (Gibco BRL) and oligo (5 μl) in 1 ml serum free media for approximately 4 hours (final oligo concentrations=5 μM). The media was then replaced with 2 ml normal medium and oligo was added to obtain a concentration of 5 μM. Medium and oligo were then replaced daily for the following 3 days. The oligos used were the following:

34: DW2-34B (antisense phosphodiester) 5' CAT CTG CCA TTC CCA CTC TA 3' (SEQ ID NO 9)

35: DW2-35C 5' Phosphorothioate of 34 (SEQ ID NO 10)

36: DW2-36C (random control phosphodiester) 5' CTG ACT GCC AAC TAT GAA CA 3' (SEQ ID NO 11)

37: DW2-37D 5' Phosphorothioate of 36 (SEQ ID NO 12)

The cells grew reasonably well, however throughout the growth period, there were less cells in the wells treated with oligo 35 than in the others and many cells in these wells were floating. Several cells were also deteched in the wells treated with oligo 37.

Experiment A

In this experiment, cells were grown in presence of the oligos for longer than in the previous experiment (14 days). The initial treatment comprised of 5 μl lipofectin and 10 μl oligo in 1 ml media. Subsequently, media was changed and oligo added (10 μl in 2 ml) daily for 9 days and for the final 4 days, to avoid losing cells that were floating but not necessarily dead, the medium was changed only once and oligo added to the medium every other day.

Cells for this experiment were slow to start growing. During the first week of treatment with oligo, cells remained quite sparse and a very high proportion of cells were observed to be round and/or floating. During the second week, as the cells started to grow more nicely, clumps of cells appeared in the control wells and in the 34 and 36. In the wells treated with oligo 35, there were consistently fewer cells and a higher proportion of floating cells than in control wells. In addition, the 35 cells that remained attached were more elongated than controls. Similar features were observed to a lesser extent in the 37 cells. Toward the end of the experiment, the control cells seemed more elongated than they had been previously, though significantly less than the 135 cells. Wells 34 and 36 contained more large clusters of cells than the others (even controls). On the whole, there were fewer clumps in wells 35 and 37 than in all the others. Oligo 34 (antisense phosphodiester) appeared to have no effect on cell morphology.

To determine whether treatment with DNA MeTase antisense oligonucleotides inhibits tumorigenesis in vitro, the ability of the treated cells to grow in an anchorage independent fashion was determined. Two sets of cells were analyzed: Set A was treated for 15 days and Set B was treated for 9 days. The number of cells were determined by inspection with the naked eye 18 days after plating. As shown in FIG. 7, the cells treated with oligo 35 have lost the ability to grow in an anchorage independent fashion in vitro, indicating inhibition of tumorigenicity in vitro.

Experiment B

Given the fact that cells did not grow very well in the initial stages of experiment A, more cells (~150,000 instead of 80,000) were plated to repeat the experiment. These cells were treated with lipofectin (5 μl) and oligo (10 μl) on day 1 and then the medium was changed and 10 μl oligo were added daily for three days and for the next four days, 10 μl oligo were added daily and the medium was changed only once.

After the 8 days of treatment, cells in wells 36 and 37 were similar in appearance to the control wells. Only the cells treated with oligo 35 looked significantly different from the others in that there had been less growth and cells appeared on the whole less "clumpy" than controls. The cells treated with oligo 35 again lost their ability to form colonies in soft agar, indicating reversal of tumorigenicity in vitro.

Dose Curve:

Cells were treated for 5 days with different doses of oligo 35 (antisense phosphorothioate): 0.5 μM, 1.5 μM, 5 μM, 15 μM and 50 μM.

Well	Initial lipofectin*	Initial oligo	Daily oligo
control	5 μl	0	0
0.5 μM	5 μl	1 μl (1 μM)	1 μl (0.5 μM)
1.5 μM	5 μl	3 μl (3 μM)	3 μl (1.5 μM)
5 μM	5 μl	10 μl (10 μM)	10 μl (5 μM)
15 μM	5 μl	30 μl (30 μM)	30 μl (15 μM)
50 μM	5 μl	100 μl (100 μM)	100 μl (50 μM)

*Lipofectin reagent (Gibco BRL)

Initial treatment with lipofectin and oligo were in 1 ml medium and subsequently, cells were in 2 ml medium.

Treatment with oligo 35 resulted in dramatic changes in cell morphology. At all doses, formation of large clusters of cells was inhibited with respect to the controls. As oligo concentration increased, cells became less clumpy and more elongated. Increasing numbers of floating cells appeared, many of which were alive as revealed by viability counts.

Upon treatment with 15 μM oligo, cells became dramatically elongated and no clumps of cells could be seen (see pictures). A high proportion of cells were floating, however viability was found to be over 50%, suggesting that many of the floating cells are still alive.

EXAMPLE 9

In Vivo Inhibition of Tumorigenicity Using Antisense Technology

In vivo inhibition of DNA methyl transferase expression and tumorigenesis can be achieved by administration of the antisense oligonucleotides of the present invention to mammals. For example, administration into a mouse can be by slow infusion pump at a rate of about 0.5–3.0 nMoles/hr (about 0.15–1.0 mg of an oligonucleotide 20-mer per kg of body weight). Alternatively, intravenous injection of about 1–5 mg of the oligonucleotide per kg body weight can be made into the tail vein. After about 10 to 21 days the tumors can be excised and analyzed for DNA methyl transferase expression as well as by observing the weight and morphology of the tumors. Tumors and DNA methyl transferase

levels of mice treated with a control oligonucleotide can be compared.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 12

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i v) ANTI-SENSE: YES

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CATCTGCCAT TCCCACTCTA 20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i v) ANTI-SENSE: YES

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTGGCATCTG CCATTCCCAC TCTA 24

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..19
(D) OTHER INFORMATION: /note="5'PRIMER BASES 154-172"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCCGAATCGG TTTCCACCC 19

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:

(A) NAME/KEY: misc_feature
(B) LOCATION: 1..21
(D) OTHER INFORMATION: /note="3'PRIMER BASES 472-492"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGAGGATGAG GGCCTGAATG C 21

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..18
(D) OTHER INFORMATION: /note="PRIMER 1-18"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGACTGGGGT GAGGACGG 18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..24
(D) OTHER INFORMATION: /note="PRIMER 620-610"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTTCAGTAGA TAACGCACTG CTGG 24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..19
(D) OTHER INFORMATION: /note="ANTI SENSE PRIMER"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCAAACAGAA TAAAGAATC 19

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:

(A) NAME/KEY: misc_feature
(B) LOCATION: 1..19
(D) OTHER INFORMATION: /note="SENSE PRIMER"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTATGGTGGT TTGCCTGGT 19

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i v) ANTI-SENSE: YES

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /note="Oligo 34: DW2-34B
(antisense phosphodiester)"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CATCTGCCAT TCCCACTCTA 20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i v) ANTI-SENSE: YES

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /note="Oligo 35: DW2-35C
(antisense phosphorothioate)"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CATCTGCCAT TCCCACTCTA 20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /note="Oligo 36: DW2-36C (Random
Control Phosphodiester)"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTGACTGCCA ACTATGAACA 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs

-continued

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..20
(D) OTHER INFORMATION: /note="Oligo 37: DW2-37D (Random Control Phosphorothioate)"

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTGACTGCCA ACTATGAACA 20

What is claimed is:

1. An antisense oligonucleotide having the sequence 5'-CATCTGCCATTCCCACTCTA-3' (SEQ ID NO: 1).

2. An antisense oligonucleotide having the sequence 5'-TTGGCATCTGCCATTCCCACTCTA-3' (SEQ ID NO: 2).

3. An antisense oligonucleotide according to claim **1**, wherein the oligonucleotide is stabilized by modifications chosen from the group consisting of methylphosphonothioate internucleotide linkages, phosphorothioate internucleotide linkages, methylphosphonate internucleotide linkages, phosphoramidate internucleotide linkages, a 3' end cap, a 3' hair-pin loop structure, and combinations thereof.

4. An antisense oligonucleotide according to claim **2**, wherein the oligonucleotide is stabilized by modifications chosen from the group consisting of methylphosphonothioate internucleotide linkages, phosphorothioate internucleotide linkages, methylphosphonate internucleotide linkages, phosphoramidate internucleotide linkages, a 3' end cap, a 3' hair-pin loop structure, and combinations thereof.

* * * * *

US005578716A

United States Patent [19]

Szyf et al.

[11] Patent Number: 5,578,716

[45] Date of Patent: Nov. 26, 1996

[54] **DNA METHYLTRANSFERASE ANTISENSE OLIGONUCLEOTIDES**

[75] Inventors: **Moshe Szyf**, Cote St. Luc, Canada; **Eric von Hofe**, Wellesley, Mass.

[73] Assignees: **McGill University**, Canada; **Hybridon, Inc.**, Worcester, Mass.

[21] Appl. No.: **161,673**

[22] Filed: **Dec. 1, 1993**

[51] **Int. Cl.**^{6} **C07H 21/00**; A61K 48/00

[52] **U.S. Cl.** **536/24.5**

[58] **Field of Search** 514/44; 536/24.5

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Primary Examiner—Jacqueline M. Stone
Assistant Examiner—D. Curtis Hogue, Jr.
Attorney, Agent, or Firm—Hale and Dorr

[57] **ABSTRACT**

The invention encompasses tumorigenicity-inhibiting antisense oligonucleotide sequences complementary to mRNA or double-stranded DNA that encodes mammalian DNA methyl transferase. It further encompasses methods for inhibiting tumorigenicity and pharmaceutical composition comprises the tumorigenicity-inhibiting antisense nucleotide.

4 Claims, 4 Drawing Sheets

In a fifth embodiment, tumorigenicity-inhibiting modified oligonucleotides are self-stabilized by having a self-complementary region that hybridizes intramolecularly with the oligonucleotide to form an exonuclease resistant hairpin-like structure (see e.g., Agrawal et al., *Nucleic Acids Res.* 20: 2729–2735 (1993). Modified oligonucleotides according to this embodiment of the invention are generally characterized by having two regions: a DNA MeTase hybridizing region and a self-complementary region. The DNA MeTase hybridizing region has a nucleotide sequence that is complementary to an essential nucleic acid sequence of DNA MeTase. Preferably, this region has from about 6 to about 100 nucleotides. In this embodiment, the oligonucleotide is stabilized, i.e., rendered resistant to exonucleolytic degradation by base-pairing between the target hybridizing region and the self-complementary region and/or by base-pairing between complementary sequences within the self-complementary region.

To directly inhibit DNA methylation in Y1 cells, either the DNA MeTase antisense expression construct pZαM or a pZEM control vector, Szyf, et al., *J. Biol. Chem.*, 267: 12831–12836 (1992)) was introduced into Y1 adrenocortical carcinoma cells by DNA-mediated gene transfer as follows.

The second significant region of self-stabilized oligonucleotides according to the invention is the self-complementary region. The self-complementary region contains oligonucleotide sequences that are complementary to other oligonucleotide sequences within the oligonucleotide. These other oligonucleotide sequences may be within the DNA MeTase hybridizing region

The complementary sequences form base pairs, resulting in the formation of a hairpin structure

will presumably have loops of 4 or more nucleotides resulting from non-base-paired nucleotides.

Of course, the intramolecular base-pairing can be so extensive as to involve every nucleotide of the oligonucleotide. Preferably, this will involve a self-complementary region of about 50 nucleotides or less.

US005631148A

United States Patent [19]

Urdea

[11] Patent Number: 5,631,148

[45] Date of Patent: May 20, 1997

[54] **RIBOZYMES WITH PRODUCT EJECTION BY STRAND DISPLACEMENT**

[75] Inventor: **Michael S. Urdea**, Alamo, Calif.

[73] Assignee: **Chiron Corporation**, Emeryville, Calif.

[21] Appl. No.: **231,227**

[22] Filed: **Apr. 22, 1994**

[51] **Int. Cl.**[6] **C12P 19/34**; C12Q 1/68; C07H 21/00; C07H 21/04

[52] **U.S. Cl.** **435/91.31**; 435/6; 435/172.1; 536/23.1; 536/23.2; 536/24.5

[58] **Field of Search** 435/6, 91.2, 91.3, 435/91.31, 91.4, 172.1, 199, 320.1; 514/44; 536/23.1, 23.2, 24.5, 25.3

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Primary Examiner—John L. LeGuyader

Assistant Examiner—Thomas G. Larson

Attorney, Agent, or Firm—Laura A. Handley; Kenneth M. Goldman; Robert P. Blackburn

[57] **ABSTRACT**

Ribozymes designed to provide improved rates of catalytic turnover are described. The compounds of this invention comprise a catalytic region, at least one substrate binding region, and at least one displaceable antisense arm, whereby the rate of release of the endonuclease cleavage fragments is enhanced. A method to make such ribozymes is also described.

28 Claims, 3 Drawing Sheets

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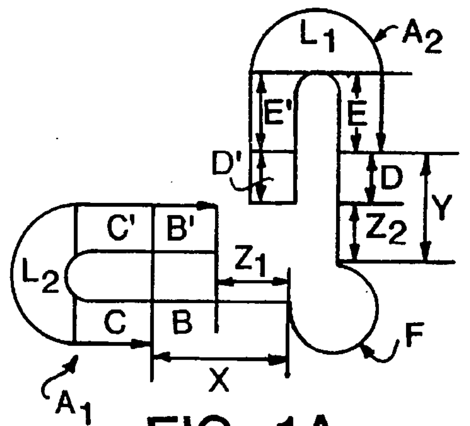

FIG. 1A

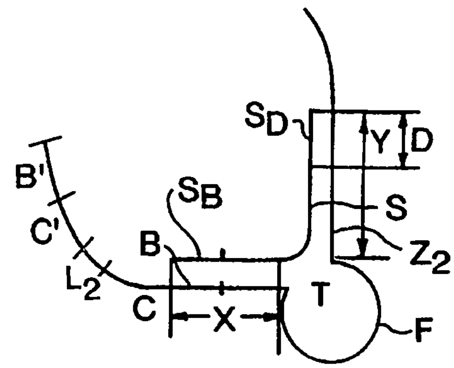

FIG. 1B

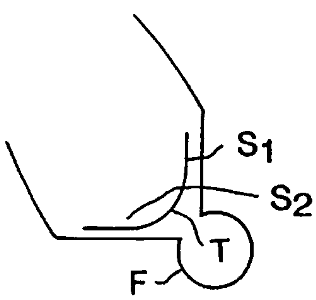

FIG. 1C

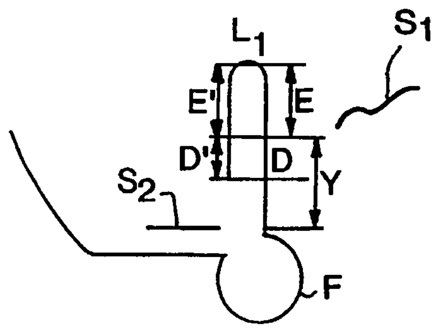

FIG. 1D

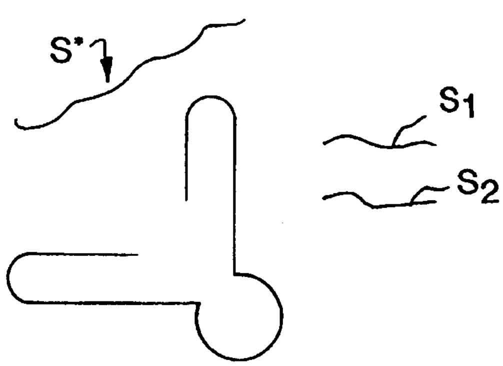

FIG. 1E

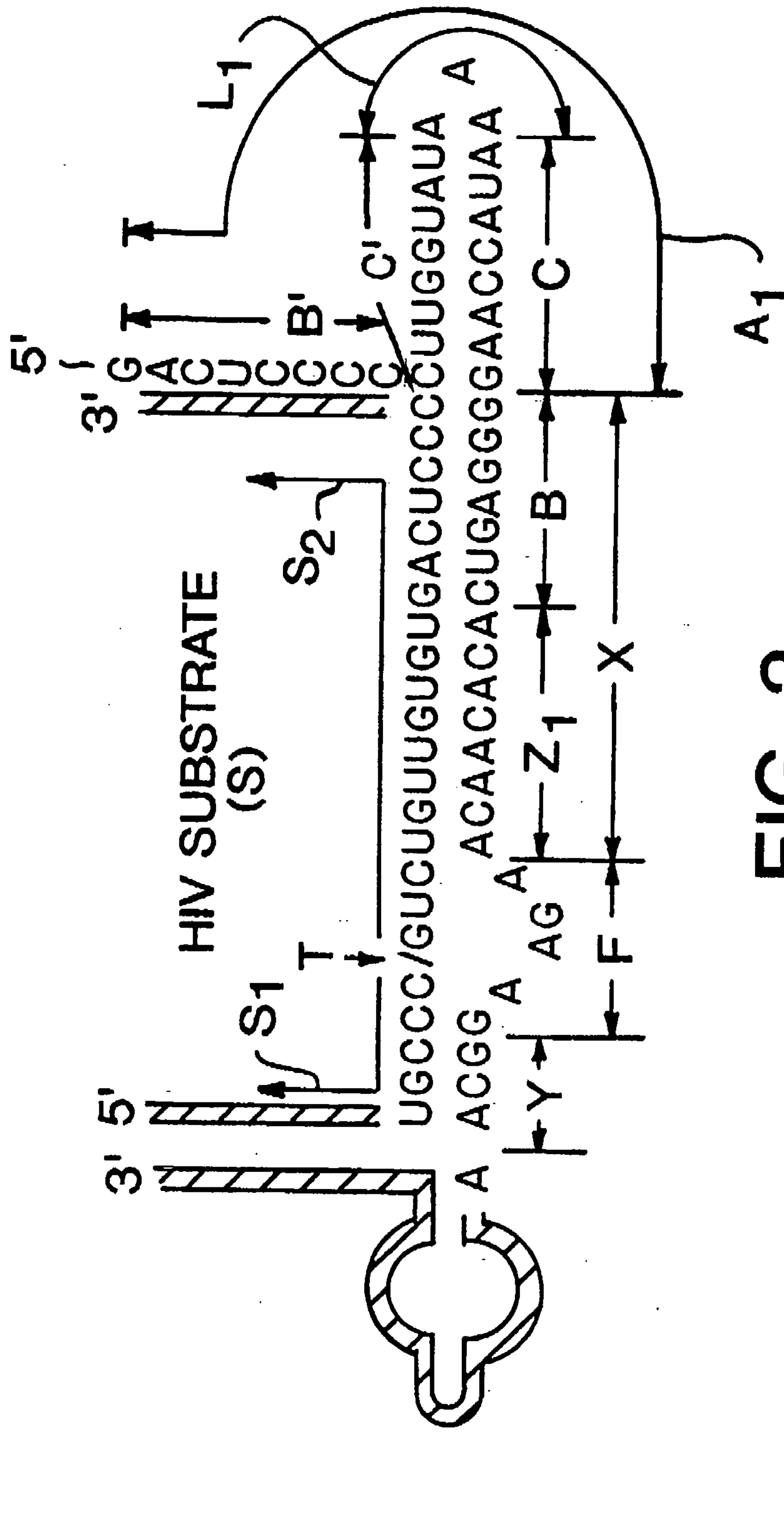
HIV SUBSTRATE
(S)
UGCCC/GUCUGUUGUGUGACUCCCCUUGGUAUA
ACGG
ACAAACACACUGAGGGGAAACCAUAAA
5'
3'
L1
A1
S1
S2
T
B'
C'
C
B
X
Z1
F
Y

FIG. 2

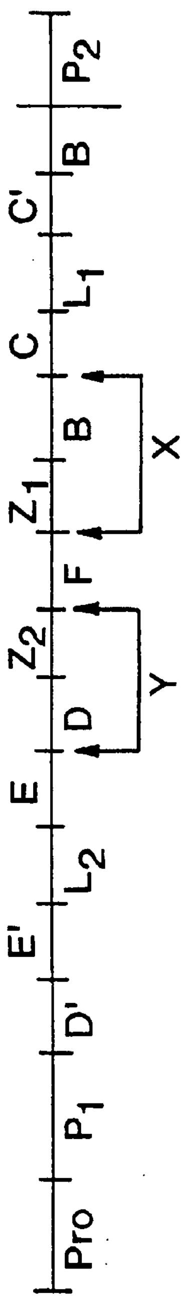
Pro
P1
D'
E'
L2
E
D
Z2
F
Z1
B
C
L1
C'
B
P2
Y
X

FIG. 3

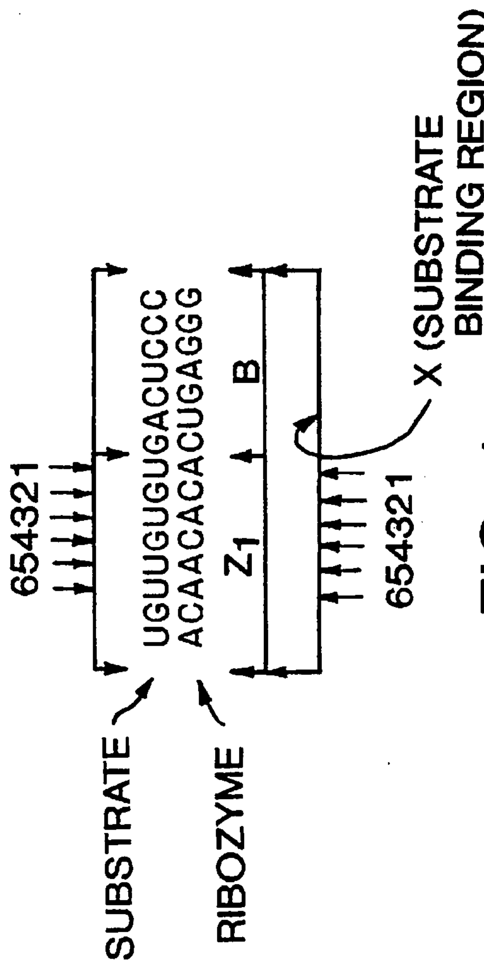
654321
UGUUGUGUGACUCCC
ACAACACACUGAGGG
Z1
B
654321
SUBSTRATE
RIBOZYME
X (SUBSTRATE BINDING REGION)

FIG. 4

RIBOZYMES WITH PRODUCT EJECTION BY STRAND DISPLACEMENT

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to compounds known as ribozymes.

Ribozymes are polynucleotides which "have the intrinsic ability to break and form covalent bonds." Symons, *Ann. Rev. Biochem.* 61:641 (1992). Of primary interest here are ribozymes which break bonds—that is, which cleave a long polynucleotide strand into two cleavage fragments. The first ribozymes were thought to act only upon RNA, but ribozymes that cleave single-stranded DNA have recently been reported. Cech et al., U.S. Pat. No. 5,180,818, the disclosure of which is incorporated by reference.

Ribozymes are valuable in vivo therapeutic agents that inactivate target RNA or DNA within the cell. In particular, ribozymes are exciting therapeutic candidates for AIDS. In vivo applications of ribozymes have been described in U.S. Pat. No. 5,254,678, U.S. Pat. No. 5,225,337, U.S. Pat. No. 5,168,053, and U.S. Pat. No. 5,144,019, the disclosures of which are incorporated by reference herein.

Ribozymes also can be efficient in vitro experimental reagents akin to restriction endonucleases, giving a researcher the ability to cleave a polynucleotide at a particular site. In vitro applications of ribozymes have been described in, e.g., U.S. Pat. No. 5,225,337, U.S. Pat. No. 5,180,818, U.S. Pat. No. 5,093,246, U.S. Pat. No. 5,037,746, and U.S. Pat. No. 4,987,071, the disclosures of which are incorporated by reference herein.

Ribozymes have the potential to serve as "catalysts" of chemical reactions, either in vitro or in vivo. In general, a catalyst will assist and/or drive the chemical reaction, without itself being altered in the process. After a catalytic event, the catalyst may be regenerated and is able to assist in another round of chemical reaction. Catalytic reactions may be more specifically described by two parameters—the specificity of a catalyst to selectively interact only with a particular substrate molecule, and the relative ability of a catalyst to alter the kinetics or rate at which a chemical reaction proceeds. Thus a ribozyme, like other catalysts such as protein-based enzymes, may be characterized in terms of both its kinetics and its specificity. Particularly useful ribozymes, like protein-based enzymes, will combine the qualities of being able to act rapidly and with good specificity.

2. Description of the Problem

The first ribozyme was described by Thomas Cech and colleagues in 1982, and was isolated from *Tetrahymena thermophila*. Kruger et al., *Cell* 31:147 (1982); U.S. Pat. No. 5,180,818; U.S. Pat. No. 5,116,742; U.S. Pat. No. 5,093,246; U.S. Pat. No. 5,037,746; U.S. Pat. No. 4,987,071. The Tetrahymena ribozyme catalyzed the excision of an intervening sequence (termed an IVS or intron) from within its own RNA, and subsequently ligated the two remaining exons. Other ribozymes of this sort, referred to as "Group I introns," were subsequently identified. Symons, *Ann. Rev. Biochem.*, p. 642. A similar class of self-splicing ribozymes have been identified and denominated "Group II introns." Id. Because the cleavage reactions of Group I and Group II ribozymes are intramolecular and result in alteration of the ribozyme itself, they cannot be described as catalytic. These ribozymes may be termed "native" ribozymes.

Another broad class of native ribozymes was discovered amongst various pathogenic plant RNAs. Long and

Uhlenbeck, *FASEB J.* 7:25–30 (1993). Many of these native ribozymes have been described as "hammerhead" ribozymes, in reference to the secondary structure which the ribozymes assume. Symons, *Ann. Rev. Biochem.*, p. 645. Specifically, the hammerhead structure comprises a highly conserved nucleotide sequence in the region of catalytic activity. The catalytic region is substantially single-stranded RNA and is flanked by three regions of helical base-pairing. The endonuclease reaction catalyzed by the hammerhead ribozymes differs from that of the Group I, Group II, and RNAase P ribozymes in that it is a transesterification reaction producing a 5' hydroxyl and a 2',3'-cyclic phosphate. The native hammerhead ribozymes undergo intramolecular cleavage, with only a single turnover for each. Symons, *Ann. Rev. Biochem.*, p. 642.

Native ribozymes having other secondary structures have also been characterized. Hampel et al., *Biochemistry* 28:4929 (1989), describe a ribozyme which displays a secondary structure referred to as "hairpin." The hairpin structure, like the hammerhead structure, catalyzes cleavage via a transesterification reaction, and with similar stereochemical properties. Symons, *Ann. Rev. Biochem.*, p. 660. Like the hammerhead structure, the hairpin structure contains regions of highly conserved sequences, with the catalytic site in close proximity to a base-paired region. Id. at 661. Other researchers have identified a ribozyme in the Hepatitis Delta Virus (HDV), and have described the structure as an "axehead." Id. at 662–64. It too contains a highly conserved region, and it too contains several base-paired regions in close proximity to a single-stranded catalytic region. Id.

Following the discovery of native, non-catalytic ribozymes, researchers discovered native ribozymes capable of intermolecular cleavage reactions. In 1983, Guerrier-Takada et al. reported that the RNA component of RNAase P could cleave its tRNA substrate, even in the complete absence of protein. *Cell* 35:849 (1983). Soon thereafter, Cech et al. reported that a fragment of Tetrahymena catalyzed a number of transesterification reactions in a truly catalytic manner. Symons, *Ann. Rev. Biochem.*, p. 642.

Subsequently, Uhlenbeck and colleagues exploited the highly conserved catalytic region and the helical flanking regions of the hammerhead structure to design the first synthetic catalytic ribozyme. Symons, *Ann. Rev. Biochem.*, p. 647. Other examples of synthetic catalytic ribozymes based on the hammerhead structure followed. E.g., U.S. Pat. No. 5,254,678; Jeffries and Symons, *Nucl. Acids Res.*, 17:1371 (1989); and Koizumi et al., *FEBS Letters* 239:285 (1988). The hairpin structure has been exploited in the formation of a synthetic ribozyme which cleaves HIV-1 RNA. Ojwang et al., *Proc. Nat. Acad. Sci.* 89:10802 (1992); U.S. Pat. No. 5,144,019. The HDV ribozyme sequence and structure also has been characterized. Perrotta and Been, *Biochemistry* 31:16–21 (1992); U.S. Pat. No. 5,225,337.

In order to be of practical value, a ribozyme must act intermolecularly on a separate substrate molecule, and remain intact so as to act on subsequent substrate molecules. Ribozymes which perform such intermolecular reactions are termed catalysts, akin to the enzymatic proteins which catalyze myriad chemical reactions within the cell.

Ribozymes, like protein-based enzymes, may be characterized by the kinetic parameters of the reactions that they catalyze. The rate of catalysis may be described by one parameter designated k_{cat}, otherwise referred to as the "turnover number." That parameter describes the rate of release of the cleaved substrate, and is measured in terms of

number of substrate molecules cleaved and released per minute. If this turnover number is low, the reaction as a whole will be slowed. The literature to date for synthetic ribozymes generally reports k_{cat} values in the range of 0.5–2.1 per minute, Symons, *Ann. Rev. Biochem.*, p. 649, although one group investigating highly modified hammerhead structures, in which the flanking side-arms of the hammerhead are entirely modified to contain DNA rather than RNA, have reported slightly higher turnover rates. Hendry et al., *Nucleic Acids Res.* 20:5737–41 (1992) (k_{cat} of 8.9 per minute). These catalytic rates are well below those of many enzymatic proteins, which are more typically in the range of 10–10.000 per minute. Zubay, *Biochemistry*, at 141. Although one review states that such low turnover rates "rival that of the typical DNA restriction enzymes," Long and Uhlenbeck, *FASEB J.* at 26, increased turnover rates would be greatly desired by those who would use ribozymes for either in vitro or in vivo uses.

The catalytic rate of ribozymes is further slowed when synthetic ribozymes are designed to incorporate larger regions of ribozyme/substrate base pairing necessary to provide rapid and stable binding in vivo. E.g., Taylor et al., *Nucleic Acids Res.* 20:4559 (1992); Heidenreich and Eckstein, *J. Biol. Chem.* 267:1904–1909 (1992); Bennett and Cullimore, *Nucleic Acids Res.* 20:831–837 (1992); Goodchild and Kohli, *Arch. Biochem. Biophys.* 284:386–91 (1991). Although such increased base pairing improves the specificity of the ribozyme catalytic reaction, once the substrate is cleaved the larger regions of base pairing inhibit the release of the cleavage fragments. Id. Thus, to date practical in vivo use of ribozymes has been inhibited by a perceived need to trade off specificity and stability, on the one hand, with rapid catalytic reactions, on the other.

Researchers have attempted to increase the in vivo efficacy of ribozymes by chemically modifying their structures to increase resistance to the natural degradative processes within the cell. A review of such modifications is provided by Heidenreich et al., *FASEB J.* 7:90–96 (1993). Despite some progress in the chemical modification of synthetic ribozymes, their practical usefulness remains limited, in part because of the low turnover number (k_{cat}) characteristic of the ribozymes known to date. This is particularly true for synthetic ribozymes which have been designed with extensive regions of substrate interaction designed to optimize the specificity of the interaction between synthetic ribozyme and substrate.

Accordingly, there exists a need for synthetic ribozymes having improved stability and rates of catalytic turnover, both for in vitro and in vivo applications.

SUMMARY OF THE INVENTION

This invention provides a synthetic catalytic ribozyme with enhanced stability and rates of product release. In general, the invention features a synthetic ribozyme polynucleotide comprising a catalytic region having endonuclease activity specific for a target polynucleotide sequence of a substrate that is linked directly or indirectly to at least one substrate binding region having a competitive binding nucleotide sequence and at least one displaceable antisense arm comprising first and second stabilization regions and a displacement region capable of forming a hybrid with the competitive binding nucleotide sequence. In one preferred embodiment of the invention, the displaceable antisense arm is a unitary, covalently linked structure. In another preferred embodiment, the displaceable antisense arm comprises a first fragment containing a first stabilization region and a second fragment containing a second stabilization region, wherein the first fragment is linked, directly or indirectly, to the first substrate binding region and the second fragment is associated only by noncovalent bonds between the first and second stabilization regions. In yet another preferred embodiment, this invention features ribozymes in which the sugar-phosphate backbone has been chemically modified.

Another aspect of this invention features a method for selecting ribozymes with enhanced rates of product release, the method comprising; constructing at least one set of synthetic ribozyme oligonucleotides comprising variable length substrate binding regions and displaceable antisense arm regions, and further comprising an inactivated catalytic site; constructing a desired substrate; contacting the set of synthetic oligonucleotides with the substrate at a temperature that is less than a preselected temperature; capturing substrate/oligonucleotide complexes and subjecting them to the preselected temperature; capturing and amplifying any oligonucleotides released at the preselected temperature; repeating these steps until a constant binding and release is found; cloning, isolating, and sequencing any oligonucleotides released after that constant binding and release is achieved; and activating the catalytic sites of any of such oligonucleotides.

In yet another aspect, this invention features a method for improving the rate of endonuclease activity of a known ribozyme having a catalytic region linked, directly or indirectly, to at least one substrate binding region, comprising; providing the ribozyme polynucleotide; obtaining a substantial portion of the nucleotide sequence of at least one substrate binding region of the ribozyme; selecting within at least one of any of the substrate binding regions a competitive binding nucleotide sequence capable of forming a first hybrid with the substrate; and modifying the ribozyme to provide at least one displaceable antisense arm linked, directly or indirectly, to the selected substrate binding region containing the competitive binding nucleotide sequence, wherein the displaceable antisense arm further comprises a first stabilization region, a second stabilization region, and a displacement region capable of forming a second hybrid with the competitive binding nucleotide sequence.

In still another aspect, this invention features a method for cleaving a target nucleotide sequence, the method comprising; providing a desired substrate; providing a synthetic ribozyme polynucleotide comprising a catalytic region having endonuclease activity specific for the target polynucleotide sequence of the substrate, at least one substrate binding region linked, directly or indirectly, to a catalytic region, with the substrate binding region further comprising a competitive binding nucleotide sequence capable of forming a first hybrid with the substrate, and at least one displaceable antisense arm linked, directly or indirectly, to the substrate binding region, with the displaceable antisense arm further comprising a first stabilization region, a second stabilization region, and a displacement region capable of forming a second hybrid with the competitive binding nucleotide sequence, and; contacting the synthetic ribozyme polynucleotide and the substrate to allow the catalytic region to cleave the substrate at its target nucleotide sequence.

The details of the invention will become apparent to those skilled in the art after having read the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 parts a–e is a schematic representation of a portion of a synthetic ribozyme polynucleotide, displaying the rela-

tion of the substrate binding region of the ribozyme to the stabilization regions and displacement regions of the displaceable antisense arm, and alternatively to the substrate. FIG. 1*a* depicts a ribozyme structure having two displaceable antisense arms, two substrate binding regions and a catalytic region. FIG. 1*b* depicts a substrate bound to the substrate binding region of the ribozyme. FIG. 1*c* depicts two bound substrate fragments after cleavage of the substrate by the ribozyme. FIG. 1*d* depicts rehybridization of one displaceable antisense arm and corresponding displacement of one substrate fragment. FIG. 1*e* depicts the rehybridization of the second displaceable antisense arm and corresponding displacement of the second substrate fragment.

FIG. 2 is a diagrammatic representation of a ribozyme of the hairpin variety, modified to contain one displaceable antisense arm.

FIG. 3 is a diagrammatic representation of a synthetic oligonucleotide used for isolating optimized synthetic ribozymes.

FIG. 4 is a diagrammatic representation of a method for optimizing a ribozyme (SEQ ID NO:1) specific for HIV (SEQ ID NO:2).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Detailed Description of the Invention

As used herein, a "ribozyme polynucleotide" is a polynucleotide that has the ability to catalyze the cleavage of a polynucleotide substrate. In general, it will have a first end and a second end, wherein the first end may be either the 5' or the 3' end of the polynucleotide. It further comprises a catalytic region, at least one substrate binding region, and at least one displaceable antisense arm. It may be classified as, but is not limited to, ribozyme structures of the hammerhead, hairpin, HDV, RNAase P, L-19 IVS, Group I, or Group II types. It may be "natural," i.e., naturally occurring in nature, "synthetic," i.e., designed and synthesized in order to bind and cleave a desired substrate, or "known," i.e., either a natural or synthetic ribozyme that has been previously sequenced and characterized.

A generalized depiction of the synthetic ribozyme polynucleotide of the claimed invention is provided in FIG. 1. Although FIG. 1 depicts a ribozyme having a hammerhead structure modified to contain two displaceable antisense arms, it is not intended to suggest that the invention is limited to this particular structure. FIG. 2 provides a diagrammatic representation of a ribozyme of the hairpin variety. Again, it is not intended to suggest that the invention is limited to this particular structure.

Substrate cleavage is performed by the "catalytic region" F of the ribozyme polynucleotide. Generally, the catalytic region will contain a region of highly conserved bases that are believed to be necessary to ensure proper interaction with the substrate. Long and Uhlenbeck, *FASEB J.* 7:25 (1993), and Symons, *Ann. Rev. Biochem.*, 61:641 (1992), provide thorough discussions of the sequence requirements of various catalytic regions. The disclosures of those references are incorporated herein in their entirety. For example, in ribozymes of the hammerhead type, the consensus sequence is reported to be 5'-CUGANGAN:NGAAAC, wherein N:N designates the first base pair of the hammerhead helix III. Id. at 646. A consensus sequence for the axehead structure is depicted at id. p. 664, FIG. 12. The hairpin ribozyme requires the sequence 5'-NN. . .NNGAA (GorC)NNNNCNNNNGAAACAN. . .3'(SEQ ID NO:3), wherein Helix 1 and Helix 4 occur at the ellipses. Long and Uhlenbeck, *FASEB J.* at 28. Alternatively, portions of the catalytic region may be provided by the substrate rather than the ribozyme. Id. at 27 FIG. 2. The catalytic region of the HDV ribozyme is reported as 5'-CCGNNCUGGG (SEQ ID NO:4). Perrotta and Been, *Biochemistry* 31:16, 17 (1992); see also U.S. Pat. No. 5,225,337, FIG. 2*b* (sequences containing delta ribozyme activity) and U.S. Pat. No. 5,225,347, FIG. 3 (proposed secondary structure of 110 nucleotide HDV subfragment possessing autocatalytic activity). The catalytic region for an RNAase P ribozyme is described in U.S. Pat. No. 5,168,053, FIG. 2, with a reported invariant 5'-NCCA region. The minimum active site for L-19 IVS ribozyme catalytic activity is described in U.S. Pat. No. 5,168,053, Col. 16, line 46, through Col. 17, line 4, and a diagrammatic representation of the catalytic site is given in FIG. 2, reporting a conserved sequence 5'-GGAGGG, which hybridized with the required substrate sequence CUCU. See also U.S. Pat. No. 5,116,742, FIG. 8 (describing interaction of G^{414} with the bound L-19 IVS ribozyme substrate).

Referring to FIG. 1*a*, an example of the structure of the ribozyme polynucleotide of this invention is now described in further detail. This diagrammatic representation is based on the highly conserved hammerhead structure described by Haseloff, U.S. Pat. No. 5,254,678, which is incorporated by reference in its entirety. However, the ribozyme structure has been modified to contain two "displaceable antisense arms," designated generally as A_1 and A_2. The displaceable antisense arms compete with the substrate for binding to the substrate binding regions, as will be described in further detail herein.

Regardless of the ribozyme's general structural classification, the ribozyme polynucleotide will have at least one "substrate binding region," designated in FIG. 1 as X, which has a competitive binding nucleotide sequence B that hybridizes with a complementary region S_B of substrate S. (In FIG. 1*a*, the ribozyme contains a second, structurally similar displacement arm A_2 and a second, analogous substrate binding region designated as Y that hybridizes with the complementary region S_D of substrate S.) Substrate binding region X is linked to the catalytic region F. Generally, it will be directly linked, for example by covalent bonds. Alternatively, it may indirectly linked, for example, by an intervening polynucleotide region, that does not inhibit the necessary spatial relation of substrate S and catalytic region F. The length of the substrate binding region X may vary, with the minimum length determined by the degree of specificity required and the maximum length determined by factors including the temperature of the reaction and the nucleotide composition of the substrate binding region. Generally, when one displacement arm is used, the substrate binding region may be 4–40 nucleotides in length, or more preferably, 8–20 nucleotides in length. A second substrate binding region with no associated displacement arm would generally be 1–12 nucleotides in length, or more preferably 5–8 nucleotides in length. If two displacement arms are used, each of the substrate binding region may be 4–40 nucleotides in length, or more preferably, 4–16 nucleotides in length.

The substrate binding region of this invention contains a "competitive binding nucleotide sequence" B that can hybridize with either the region S_B of substrate S or with the "displacement region" B' of the displaceable antisense arm A_1. The displacement region B' is a nucleotide sequence that is complementary with the competitive binding nucleotide sequence B of the substrate binding region X (and thus is

substantially similar to regions$_B$ of substrates). The length of the substrate binding region and the displacement region may vary. Generally, when one displacement arm is used, the displacement region and the competitive binding nucleotide sequence may be 2–20 nucleotides in length, or more preferably, 4–10 nucleotides in length. If two displacement arms are used, each of the substrate binding region generally may be 2–20 nucleotides in length, or more preferably, 4–10 nucleotides in length.

The displaceable antisense arm A_1 comprises the displacement region B', a first "stabilization region" C, a second stabilization region C', and optionally may contain a nonhybridizing region L_1. The first stabilization region is linked, either directly or indirectly, to the substrate binding region. The first stabilization region C is substantially complementary to a second stabilization region C' of the displaceable antisense arm, and the two regions will hybridize in the absence of bound substrate. As can be seen in FIG. 1*a*, a nucleotide or sequence of nucleotides L_1 may intervene between the first and second stabilization regions. L_1 also may use some other covalent linking means, for example an ethylene glycol linker. Alternatively, if the first and second stabilization regions provide a hybridization region of sufficient length so as to not completely dissociate from one another upon substrate binding, L_1 is not required. The length of the first and second stabilization regions may vary, but generally will be within the range of 1–100 nucleotides, or, more preferably, 2–20 nucleotides.

The polynucleotide substrate S will contain a "target polynucleotide sequence" T, which is defined as a sequence that is cleaved by the catalytic region of a ribozyme. Substrate S has regions S_B and S_D that hybridize with the corresponding competitive binding nucleotide sequences B and D within substrate binding regions X and Y, respectively. Accordingly, S_B and S_D are substantially similar to displacement regions B' and D' of displaceable antisense arms A_1 and A_2.

In many instances, substrate S will be a known polynucleotide sequence. For example, Ojwang et al. reported use of a hairpin ribozyme cleaving the N*GUC sequence (in which GUC is described as a required sequence and cleavage occurs at *) to target and cleave the 5' leader sequence of HIV-1 at the position +111/112 relative to the transcription initiation site. *Proc. Natl. Acad. Sci. USA* 89:10802–10806 (1992). Rossi et al., U.S. Pat. No. 5,144,019, the disclosure of which is incorporated herein in its entirety, describes the use of hammerhead ribozymes recognizing the sequence GAAAC(X)$_n$GU, in which X is any nucleotide and n may have any value. See Col. 2, lines 39–50.

Once a target polynucleotide sequence is chosen, a ribozyme containing at least one displaceable antisense arm can easily be designed using known ribozyme consensus sequence information and base pairing rules. For example, a target substrate such as Hepatitis C virus may contain the target polynucleotide sequence 5'NNGUC*NNN3', wherein GUC is the consensus sequence for a ribozyme of the hammerhead type, and * indicates the cleavage site. Symons, *Ann. Rev. Biochem. at* 646. A synthetic ribozyme of the hammerhead type is then designed. First, it provides the consensus catalytic region 5'. . .CUGANGA. . .GAAAC. . .3'(SEQ ID NO:5), wherein the non-conserved helical regions are designated by ellipses. Id. Using conventional base pairing rules, the ribozyme is then designed to provide nucleotides flanking the 5' and 3' sides of the catalytic region that are complementary to the nucleotides flanking the conserved GUC* target sequence of the substrate. These two flanking regions of the ribozyme are the "substrate binding regions." Thus, the substrate binding regions are designed to align the 3'AC terminal portion of the hammerhead consensus catalytic region to align with the complementary, conserved GU region of the substrate, thus spatially orienting the substrate for cleavage. Id. Finally, the ribozyme is designed to provide at least one displaceable antisense arm. To do so, one first designates a portion of the substrate binding region distal from the catalytic region (i.e., either the 5' or the 3' end of the substrate binding region) as the "competitive binding nucleotide sequence," and designs a "displacement region" that is substantially similar to the substrate region and thus would hybridize with the ribozyme in that region. The remainder of the displaceable antisense arm then simply is designed to contain two regions that hybridize with one another (the first and second "stabilization regions"), with an optional linking region between the two stabilization regions if those regions would otherwise dissociate upon ribozyme binder of substrate. The first stabilization region is linked to the substrate binding region. The end result is a molecule that is designed to base pair with itself in the absence of substrate, but to dissociate and allow the substrate to bind to the ribozyme. When the ribozyme cleaves the substrate, the two resultant substrate fragments then dissociate, or are "ejected," by the rehybridization of the first and second stabilization regions and of the substrate binding region and the displacement region.

Variations on this basic description are suggested by the literature. For example, in some instances the substrate itself may provide a portion of the consensus catalytic region. Jeffries and Symons, *Nucleic Acids Res.* 17:1371, 1373 (1989). Alternatively, the ribozyme may be constructed of two or more separate oligonucleotides that base pair in the correct orientation to provide the requisite catalytic region consensus sequence. Id.

The kinetic process of ribozyme binding, cleavage, and displacement of substrate molecules is depicted in FIG. 1. In FIG. 1*a*, the representative synthetic ribozyme polynucleotide is shown in the absence of substrate. FIG. 1*b* depicts the substrate S bound to substrate binding regions X and Y and the dissociation of the displacement regions B' and D' from competitive binding nucleotide sequences B and D. FIG. 1*c* depicts the substrate S cleaved at target nucleotide sequence T, yielding two "cleavage fragments" S_1 and S_2. FIG. 1*d* depicts rehybridization of the first and second stabilization regions E and E', the hybridization of displacement region D' of displaceable antisense arm A_2 to the competitive binding nucleotide sequence D of substrate binding region Y and the corresponding displacement of cleavage fragment S_1. FIG. 1*e* depicts the analogous dissociation of cleavage fragment S_2, thereby preparing the synthetic ribozyme polynucleotide to bind to and cleave the next substrate molecule S*.

FIG. 2 represents a hairpin ribozyme (SEQ ID NO:6) designed to cleave the 5' untranslated leader sequence of HIV (SEQ ID NO:7). The ribozyme has been modified to contain one displaceable antisense arm A_1. The substrate binding region X again contains the competitive binding nucleotide sequence B, and is linked to an exemplary first stabilization region C. The exemplary second stabilization region C', which hybridizes with the first stabilization region C, is linked to C with a short intervening nucleotide sequence L_1. Displacement region B', which is substantially identical to the S_B region of the HIV substrate S, may hybridize with the competitive binding nucleotide sequence B. The ribozyme also contains a second substrate binding region Y.

Referring generally to FIG. 1, the displaceable antisense arm is designed to enhance the rate of cleavage fragment

release because the stability of the hybrid formed by the first and second stabilization regions (C/C') and by the competitive binding nucleotide sequence and displacement region (B/B') is less than that of the hybrid formed by the substrate binding region X and the substrate S, but greater than that of the substrate binding region X and the cleavage fragment of the substrate, S_2.

In general, the synthetic polynucleotide ribozyme may have one or more substrate binding regions, and one or more displaceable antisense arms. A synthetic ribozyme polynucleotide containing more than one substrate binding region need not have a corresponding displaceable antisense arm for each region. However, each substrate binding region may only have a single corresponding displaceable antisense arm.

THE DESIGN OF SYNTHETIC RIBOZYMES WITH ENHANCED RATES OF CLEAVAGE FRAGMENT RELEASE

As those in the art appreciate, the general approach diagrammed in FIG. 1 readily adapts itself to many structural variations. For example, the synthetic ribozyme polynucleotide of this invention may be one contiguous polynucleotide sequence, in which case the ribozyme is referred to as a "unitary" molecule. Alternatively, the ribozyme may be made of two or more polynucleotide sequences that hybridize to form the functional ribozyme. In such a case, the synthetic ribozyme is said to be comprised of ribozyme "fragments." Preparation and use of such ribozyme fragments in a hammerhead structure are described by Koizumi et al., *Nucleic Acids Res.* 17:7059–7071 (1989). Preparation and use of ribozyme fragments in a hairpin structure are described by Chowrira and Burke, *Nucleic Acids Res.* 20:2835 (1992). Both unitary molecules and ribozyme fragments are within the scope of this invention. Another structural variation on the general approach is to provide more than one displaceable antisense arm, as depicted in FIG. 1, in order to further facilitate the release of the substrate cleavage fragments from each of the corresponding substrate binding regions.

The invention described herein is applicable to a wide variety of ribozyme structures, as will be evident to those skilled in the art. For example, a hammerhead ribozyme is depicted diagrammatically in FIG. 1. A hairpin ribozyme structure with an added displaceable antisense arm is depicted diagrammatically in FIG. 2. The invention may also be applied to other ribozyme structures, including without limitation the Hepatitis Delta Virus ribozyme described by Robertson et al., U.S. Pat. No. 5,225,337 (which reference is incorporated herein in its entirety), the Tetrahymena L-19 IVS RNA described by Cech et al., U.S. Pat. No. 5,116,742 (which reference is incorporated herein in its entirety), and the RNAase P ribozyme described in Altman, U.S. Pat. No. 5,168,053 (which reference is incorporated herein in its entirety). Particularly, the selected ribozyme structure will have at least one substrate binding region, wherein the substrate binding region can be modified to add a displaceable antisense arm. The substrate binding region may be modified by linking the displacement arm in linear arrangement, or alternatively, may be prepared as a branched structure. E.g., Horn and Urdea, *Nucleic Acids Res.* 17:6959–67 (1989).

The basic structure of the ribozymes may also be chemically altered in ways quite familiar to those skilled in the art. For example, the 2' hydroxyl of the ribose moiety may be chemically altered. Heidenreich et al., *FASEB J.* at 92. In particular, this location may be selectively modified with O-methyl or O-allyl groups. Shibahara et al., *Nucleic Acids Res.* 17:239 (1989), Paollella et al., *EMBO J.* 11:1913 (1992). Pieken et al., *Science* 253:314 (1991), modified the 2' position with 2'-amino and 2'-fluoro groups. Many researchers also have investigated the effect of substituting 2'-deoxynucleotides at a variety of positions. E.g., Williams et al., *Proc. Nat'l Acad. Sci. USA* 89:918 (1992); Fu et al., *Proc. Nat'l Acad. Sci. USA* 89:3985 (1992); Olsen et al., *Biochemistry*, 30:9735 (1991); Yang et al., *Biochemistry* 31:5005 (1992); Perreault et al., *Nature* 344:565 (1990). Alternatively, arabinose-based nucleotides may be substituted for ribose-based nucleotides.

Although not all ribose moieties may be modified at the 2' position without adverse effect on catalytic ability, the literature provides extensive guidance to those skilled in the art as to which positions should remain unchanged. For example, using the standard hammerhead numbering system, *Nucleic Acids Res.* 20:3252 (1992), deoxynucleotide substitution at the G^9, A^{13}, and U^7 significantly decreased catalytic activity in ribozymes of the hammerhead structure. Perreault et al., *Biochemistry* 30:4020 (1991). Fu et al., supra, reported a drastic decrease of cleavage efficiency for hammerhead ribozymes substituted at the G^{10} or G^{13} position. Williams, supra, reported that substitution of the non-conserved nucleotides within a hammerhead ribozyme caused little alteration in catalytic ability. Two groups have described a hammerhead ribozyme in which the hybridizing regions are entirely composed of 2'-deoxynucleotides, and which display a significant increase in the catalytic rate. Hendry et al., *Nucleic Acids Res.* 20:5737 (1992); Taylor et al., *Nucleic Acids Res.* 20:4559 (1992).

Alternatively, the internucleotidic phosphate groups of the ribozyme may be selectively replaced with phosphorothionate. Heidenreich, supra, at 90–92. Substitution with thiophosphates 5' to all guanosines, cytidines, and uridines were reported to have little effect on the catalytic rate of the ribozyme, while substitution of multiple adenosines significantly decreased the catalytic rate. Chowrira and Burke, *Nucleic Acids Res.* 20:2835 (1992). Slim and Gait, *Nucleic Acids Res.* 19:1183 (1991), described a method of chemically synthesizing oligoribonucleotides containing a single phosphorothionate linkage in a defined stereochemical position. Ruffner and Uhlenbeck, *Nucleic Acids Res.* 18:6025 (1990), identified four phosphates in the conserved core of a hammerhead ribozyme which cannot be modified without large reductions in cleavage rates.

METHODS OF MAKING SYNTHETIC RIBOZYMES WITH ENHANCED RATES OF CLEAVAGE FRAGMENT RELEASE

Synthetic ribozymes may be synthesized directly as RNA using commercially available compounds on an automated synthesizer. In the event that a sequence is too long for efficient direct synthesis, two fragments may be joined by RNA ligase methods. Alternatively, the DNA encoding for the desired ribozyme may be designed and constructed by standard recombinant DNA techniques well known to those skilled in the art. E.g., Maniatis et al. (1989), *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Labs, Cold Spring Harbor, N.Y.).

The design and construction of an HIV-specific ribozyme of the hairpin type is used by way of example. The ribozyme is based upon the minimum catalytic center of the negative strand of the tobacco ringspot virus. Hampel and Tritz, *Biochemistry* 28, 4929–4933 (1989). See Ojwang et al.,

Proc. Natl. Acad. Sci. USA 89:10802–10806 (1992), the disclosure of which is incorporated by reference in its entirety. Based on the clone HXB2, Ratner et al., *Nature* 313:277–284 (1985), a N*GUC target sequence is located on the 5' leader sequence of HIV-1 at the position +111/112 relative to the transcription initiation site, within the sequence UGCCCGUCUGUUGUGU (SEQ ID NO:8) of the clone HXB2. FIG. 2 shows the incorporation of this sequence into a hairpin ribozyme that has been modified to contain a model displacement arm.

The ribozyme of FIG. 2 may be constructed as follows. Double-stranded oligodeoxyribonucleotides containing the desired ribozyme sequences are chemically synthesized, with each end being flanked by a suitable restriction endonuclease site. The oligonucleotides are then cloned into a corresponding suitable plasmid. Correct clones are identified by filter hybridization and confirmed by DNA sequencing. DNA fragments are cloned into a plasmid containing a suitable promotor, for example the human β-actin promoter or the adenovirus VA1 gene promotor. Yu et al., *Proc. Natl. Acad. Sci. USA* 89:6340–6344 (1993). The gene containing the DNA encoding for the ribozyme is then expressed.

In order to increase stability or alter properties of the model HIV ribozyme, ribonucleotides in catalytically non-critical positions can be replaced by deoxyribonucleotides, modified ribonucleotides (e.g., 2'-O-methyl), or non-nucleotidic components. Methods for making such chemical modifications are familiar to those skilled in the art, as exemplified by the references collected and summarized in Heidenreich et al., *FASEB J.* 7:90–96 (1993).

Alternatively, a protocol which provides for in vivo production of the ribozyme may be employed. For example, DNA encoding the desired ribozyme may be chemically synthesized and cloned into suitable plasmids. A suitable promotor, e.g. the human $tRNA^{Val}$ promoter and adenovirus VA1 promoter, may then be cloned into the plasmid upstream of the ribozyme. The plasmid may then be digested, inserted into a suitable retroviral vector, and transfected into the target cell. Yu et al. (1993), supra.

A similar strategy may be employed by selecting a suitable cleavage site in the 5' untranslated region of the Hepatitis C virus. See Cha et al., *Proc. Nat'l. Acad. Sci. USA* 89:7144 (1992) and Cha et al., *J. Clin. Microbiol.* 29:2528 (1991). A known ribozyme, e.g., the nuclease resistant chimeric ribozyme of Shimayama and Nishikawa, *Nucleic Acids Res.* 21:2605 (1993), may be modified to contain a displaceable antisense arm that facilitates release of the Hepatitis C virus cleavage products.

METHOD OF SELECTING SYNTHETIC RIBOZYME WITH OPTIMIZED RATES OF CLEAVAGE FRAGMENT RELEASE

Synthetic ribozymes having optimized rates of cleavage and turnover may be selected using repeated cycles of in vitro selection and amplification. In vitro selection and amplification of large pools of sequences with the desired properties has been shown to be useful for the isolation of such molecules. Bartel and Szostak, *Science* 261:1411–1418 (1993). In a modification of that method, a large pool of compounds with potentially beneficial ribozyme activity will initially be made as described above, but with inactive catalytic sites.

Referring to FIG. 3, two sets of synthetic oligonucleotides with variable lengths for each of the two displacement arm regions Z_1, B, C, L_1, C', B' and Z_2, D, E, L_2, E', D', along with catalytic region F, are constructed. (The letter designations B–F correspond to the representative ribozyme of FIG. 1.) "Pro" represents a promoter, preferably T_7. P_1 and P_2 represent PCR primer sites, generally comprising 15–20 bases each. Using probable ranges disclosed herein, variable length oligonucleotides can be produced by one of two ways: a) by removing a portion of the solid phase after each step of the variable portion of the synthesis, then recombining; or b) by using levulinic anhydride for capping, then removing with hydrazine:acetic acid:pyridine:H_2O. After the variable position is completed, the synthesis is continued. See e.g., Horn and Urdea, *Tetrahedron Letters* 27:2933–2937 (1986), and *Nucleic Acids Res. Symposium Series,* 16:153 (1985). Then, a total ribozyme transcription element is constructed, whereby Pol 1 and nucleotide triphosphates are filled in, ligated, and transcribed. The substrate RNA complementary to $D/Z_2/Z_1/B$ is then constructed either synthetically or enzymatically. Biotinylated nucleotides are incorporated. The transcribed ribozyme pool and the substrate are then combined at a lower temperature than desired for product release, e.g., 10°–15 C.° below physiological temperature. The complexes are captured on streptavidin beads, washed, and then subjected to the desired temperature, e.g., physiological temperature. Using $ProP_1$ and P_2' PCR primers, the released material is amplified. The process of transcription, binding, and release is repeated until a constant binding and release is found. The released product is then cloned using P_1 and P_2' PCR primers adapted with restriction sites for cloning into a T_7 promoter-containing vector. The clones are isolated and sequenced. Using in vitro mutagenesis techniques well known to those skilled in the art, the inactive catalytic sites are converted to active catalytic sites. Finally, the specific ribozymes are tested for K_{cat} and functionality.

This protocol may be used to produce the HIV-specific ribozyme, discussed above. As a first attempt, the length and composition of C, C', F would be maintained while the length of Z_1, B, and B' would be altered. For instance, the designs shown in FIG. 4 can be constructed, wherein FIG. 4 corresponds to region A_1 of FIG. 2. The numerals **1**,**2**,**3**,**4**,**5**,**6** indicate the change in junction between Z_1 and the substrate. So, in design **1** the tail of the ribozyme, B', has the sequence GACUCCC (7 bases); in design **2**, B' is UGACUCCC (8 bases) and Z_1 is shorter by one base, A. The efficiencies of each design can be assessed (i.e., K_{cat} and K_M studies). E.g., Hampel et al., *Nucleic Acids Res.* 18:299–304 (1990). Procedures for construction, characterization, and in vitro transcription of the ribozyme are given in Ojwang et al. (1992), and are discussed above.

METHODS OF USING SYNTHETIC RIBOZYMES WITH ENHANCED RATES OF CLEAVAGE FRAGMENT RELEASE

a) In vitro uses:

In vitro uses of ribozymes have been well described by Altman et al., U.S. Pat. No. 5,168,053, Cech et al., U.S. Pat. No. 5,116,742, Robertson et al., U.S. Pat. No. 5,225,337, and Haseloff, U.S. Pat. No. 5,254,678, all of which are incorporated by reference. The ribozymes described herein are interchangeable in those protocols, but will provide enhanced catalytic rates. In addition, for in vitro protocols involving ribozymes which are unstable or difficult to synthesize, it will be advantageous to complete the protocol with a more efficient ribozyme.

b) In vivo uses:

Ribozymes show great therapeutic promise for altering viral replication in vivo. Such uses have been discussed in, e.g., Altman et al., U.S. Pat. No. 5,168,053, Robertson et al.,

U.S. Pat. No. 5,225,337, and Haseloff, U.S. Pat. No. 5,254,678, all of which are incorporated by reference. The therapeutic ribozyme is exposed to the target polynucleotide in one of two general ways. First, the ribozyme may be isolated in the laboratory and packaged in a suitable delivery vehicle, for example liposomes. Taylor et al., *Nucleic Acids Res.* 20:4559 (1992). Such exogenous therapeutic approaches generally require that the ribozyme be modified to stabilize against degradation. Second, the DNA encoding the ribozyme of interest is incorporated into a vector with a suitable promotor, and delivered to the target cell. Such an endogenous delivery technique is described in Ojwang et al. (1992) and Yu et al. (1993), for example. Variations in both the endogenous and exogenous delivery techniques are familiar to those skilled in the art.

The biological activity of the ribozyme can be assessed in several ways. In vivo studies for anti-HIV activity can be conducted using transcribed or isolated HIV RNA. Transient transfection systems have been described. Ojwang et al. (1992). HIV-infected cells can be employed to study intracellular localization and efficiency of ribozymes. Appropriate animal models such as the SCID mouse, the green tail macaque, or the chimpanzee can be explored.

The stability and distribution of the ribozyme could be modified by use of targeting agents such as liposomes, conjugates targeting hepatocytes such as described by Wu et al., *J. Biol. Chem.* 267:12436 (1992), or cholesterol modifications. E.g., Grayaznov et al., *Nucleic Acids Res.* 21:5909 (1993).

In light of the above description, it is anticipated that alterations and modifications thereof will be apparent to those skilled in the art. Such other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 8

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: RNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGAGUCACA CAACA 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: RNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

UGUUGUGUGA CUCCC 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: RNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

NNNNGAASNN NNCNNNNGAA ACAN 24

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCGNNCUGGG 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: A
(B) LOCATION: 7
(D) OTHER INFORMATION: /label=variable
/ note= an intervening sequence Nx of any length may be inserted between nucleotides 7 and 8

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CUGANGAGAA AC 12

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GACUCCCCUU GGUAUAAAAU ACCAAGGGGA GUCACACAAC AAGAAGGCAA 50

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

UGCCCGUCUG UUGUGUGACU CCC 23

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

UGCCCGUCUG UUGUGU 16

What is claimed is:

1. A synthetic ribozyme polynucleotide having a first end and a second end, comprising from said first end to said second end:

a catalytic region having endonuclease activity specific for a target polynucleotide sequence of a substrate;

a first substrate binding region linked to said catalytic region, said first substrate binding region comprising a first competitive binding nucleotide sequence capable of forming a first hybrid with said substrate; and

a first displaceable antisense arm linked to said first substrate binding region, wherein said first displaceable antisense arm comprises a first stabilization region and a second stabilization region, said first stabilization region being capable of hybridizing with said second stabilization region, and a first displacement region capable of forming a second hybrid with said competitive binding nucleotide sequence.

2. The synthetic ribozyme polynucleotide of claim 1 wherein said synthetic ribozyme is a unitary molecule.

3. The synthetic ribozyme polynucleotide of claim 1 wherein said first displaceable antisense arm comprises a first fragment containing said first stabilization region and a second fragment containing said second stabilization region, wherein said first fragment is linked to said first substrate binding region, and wherein said second fragment is associated only by noncovalent bonds between said first and said second stabilization regions.

4. The synthetic ribozyme polynucleotide of claim 1 wherein said first stabilization region and said first competitive binding nucleotide sequence are separated by at least one nucleotide.

5. The synthetic ribozyme polynucleotide of claim 1, further comprising a second substrate binding region linked to said catalytic region at said first end.

6. The synthetic ribozyme polynucleotide of claim 5, further comprising a second displaceable antisense arm linked to said second substrate binding region.

7. The synthetic ribozyme polynucleotide of claim 1 wherein said first substrate binding region is directly linked by covalent bonds to said catalytic region.

8. The synthetic ribozyme polynucleotide of claim 1 wherein said first displaceable antisense arm is directly linked by covalent bonds to said first substrate binding region.

9. The synthetic ribozyme polynucleotide of claim 1 wherein said first substrate binding region is indirectly linked by an intervening polynucleotide region to said catalytic region.

10. The synthetic ribozyme polynucleotide of claim 1 wherein said first displaceable antisense arm is indirectly linked by an intervening polynucleotide region to said first substrate binding region.

11. The synthetic ribozyme polynucleotide of claim 1 wherein said synthetic ribozyme polynucleotide is of the hammerhead structure.

12. The synthetic ribozyme polynucleotide of claim 1 wherein said synthetic ribozyme polynucleotide is of the hairpin structure.

13. A composition which comprises the synthetic ribozyme polynucleotide of claim 1 in a carrier.

14. A synthetic ribozyme polynucleotide comprising at least one displaceable antisense arm, wherein the sugar-phosphate backbone of said synthetic ribozyme polynucleotide has been chemically altered.

15. The synthetic ribozyme polynucleotide of claim 14 wherein at least one ribonucleotide is replaced with a deoxynucleotide.

16. The synthetic ribozyme polynucleotide of claim 14 wherein at least one ribonucleotide is modified at the 2'-position.

17. The synthetic ribozyme polynucleotide of claim 16 wherein said at least one ribonucleotide is selected from a group consisting of 2'-fluoro, 2'-amino, 2'-O-alkyl, and 2'-O-allyl.

18. The synthetic ribozyme polynucleotide of claim 17 wherein said at least one ribonucleotide is 2'-O-methyl.

19. The synthetic ribozyme polynucleotide of claim 14 wherein selected ribonucleotides of said sugar-phosphate backbone are phosphorothionated.

20. A method for producing synthetic ribozyme polynucleotides, comprising the steps of:

constructing at least one set of synthetic ribozyme oligonucleotides comprising variable length substrate binding regions and displaceable antisense arm regions, and further comprising an inactivated catalytic site;

constructing a desired substrate;

contacting said set of synthetic oligonucleotides with said substrate at a temperature which is less than a preselected temperature;

capturing complexes of said substrate and said oligonucleotides;

subjecting said complexes to said preselected temperature;

capturing and amplifying any oligonucleotides released at said preselected temperature;

repeating the steps of constructing synthetic ribozyme oligonucleotides, contacting with substrate, capturing complexes, subjecting complexes to said preselected temperature, and capturing and amplifying released complexes, until a constant binding and release is found;

cloning, isolating, and sequencing any oligonucleotides released after said constant binding and release is achieved; and

activating the catalytic sites of any oligonucleotides released after said constant binding and release is achieved.

21. A method of cleaving a target nucleotide sequence of a substrate, comprising the steps of:

providing said substrate;

providing a synthetic ribozyme polynucleotide having a first end and a second end, comprising from said first end to said second end a catalytic region having endonuclease activity specific for said target polynucleotide sequence and a first substrate binding region linked to said catalytic region, said substrate binding region further comprising a first competitive binding nucleotide sequence capable of forming a first hybrid with said substrate, and a first displaceable antisense arm linked to said substrate binding region, said first displaceable antisense arm further comprising a first stabilization region, a second stabilization region, and a first displacement region capable of forming a second hybrid with said first competitive binding nucleotide sequence; and

contacting said synthetic ribozyme polynucleotide and said substrate to allow said catalytic region to cleave said substrate at said target nucleotide sequence.

22. The method of claim 21 wherein said synthetic ribozyme polynucleotide is a unitary molecule.

23. The method of claim 21 wherein said first displaceable antisense arm comprises a first fragment containing said first stabilization region and a second fragment containing said second stabilization region, wherein said first fragment is linked to said first substrate binding region, and wherein said second fragment is associated only by noncovalent bonds between said first and said second stabilization regions.

24. The method of claim 21 wherein said first stabilization region and said first competitive binding nucleotide sequence are separated by at least one nucleotide.

25. The method of claim 21, further comprising a second substrate binding region linked to said catalytic region at said first end.

26. The method of claim 25, further comprising a second displaceable antisense arm linked to said second substrate binding region.

27. The method of claim 21 wherein said synthetic ribozyme polynucleotide is of the hammerhead structure.

28. The method of claim 21 wherein said synthetic ribozyme polynucleotide is of the hairpin structure.

* * * * *

US005631148A

United States Patent [19]

Urdea

[11] **Patent Number: 5,631,148**

[45] **Date of Patent: May 20, 1997**

[54] **RIBOZYMES WITH PRODUCT EJECTION BY STRAND DISPLACEMENT**

[75] Inventor: **Michael S. Urdea**, Alamo, Calif.

[73] Assignee: **Chiron Corporation**, Emeryville, Calif.

[21] Appl. No.: **231,227**

[22] Filed: **Apr. 22, 1994**

[51] **Int. Cl.**^{6} **C12P 19/34**; C12Q 1/68; C07H 21/00; C07H 21/04

[52] **U.S. Cl.** **435/91.31**; 435/6; 435/172.1; 536/23.1; 536/23.2; 536/24.5

[58] **Field of Search** 435/6, 91.2, 91.3, 435/91.31, 91.4, 172.1, 199, 320.1; 514/44; 536/23.1, 23.2, 24.5, 25.3

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[57] **ABSTRACT**

Ribozymes designed to provide improved rates of catalytic turnover are described. The compounds of this invention comprise a catalytic region, at least one substrate binding region, and at least one displaceable antisense arm, whereby the rate of release of the endonuclease cleavage fragments is enhanced. A method to make such ribozymes is also described.

28 Claims, 3 Drawing Sheets

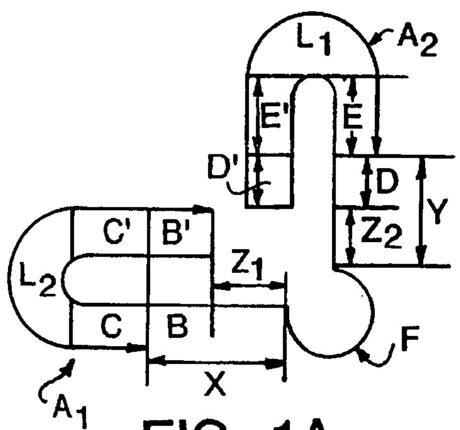

FIG. 1A

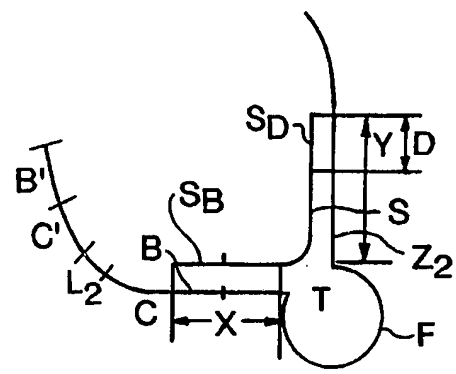

FIG. 1B

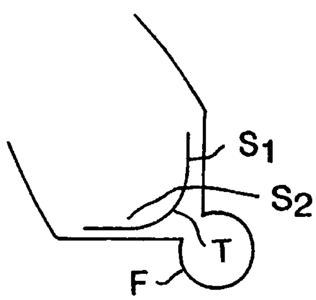

FIG. 1C

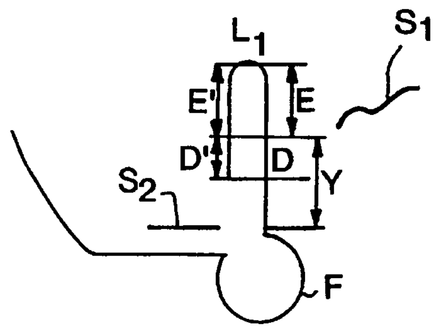

FIG. 1D

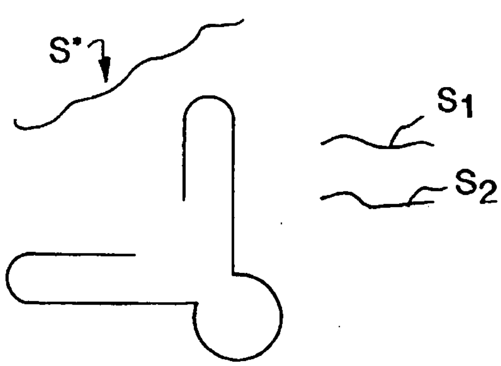

FIG. 1E

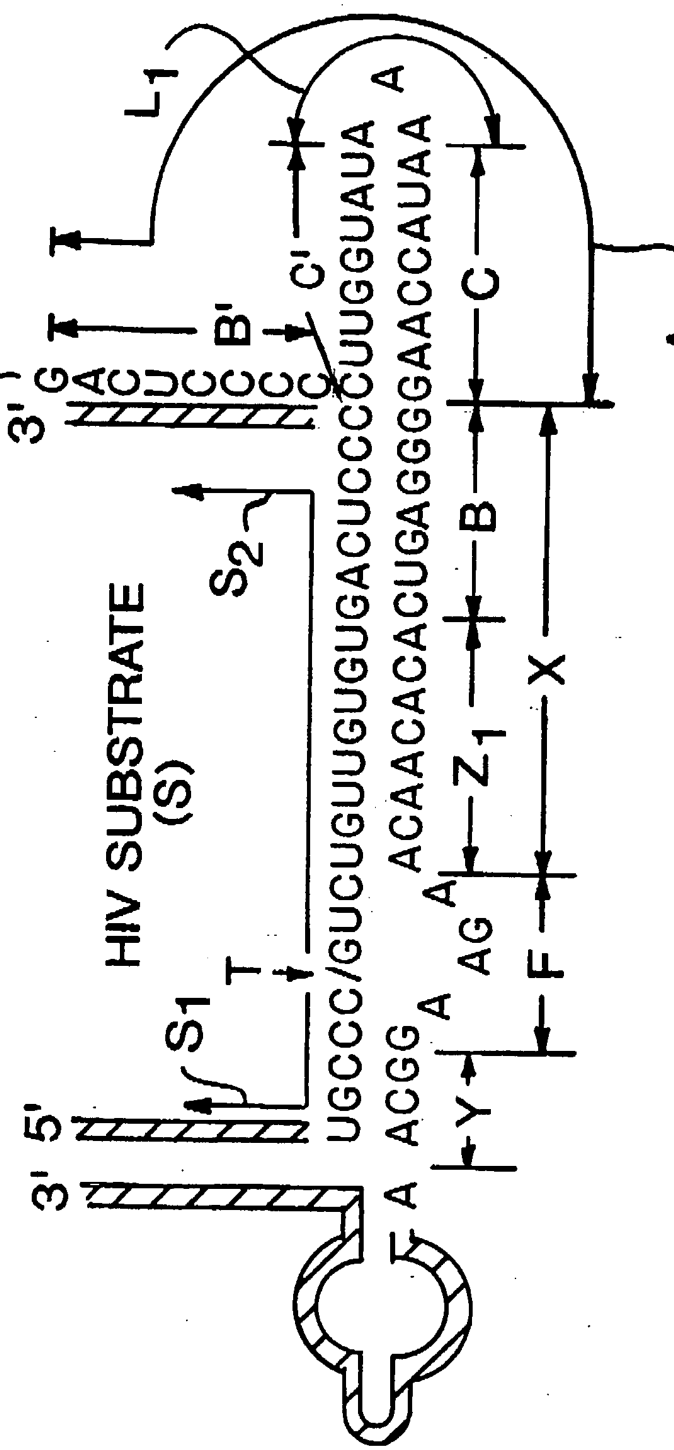

FIG. 2

the ribozyme polynucleotide will have at least one "substrate binding region," designated in FIG. 1 as X, which has a competitive binding nucleotide sequence B that hybridizes with a complementary region S_B of substrate S.

The length of the substrate binding region X may vary, with the minimum length determined by the degree of specificity required and the maximum length determined by factors including the temperature of the reaction and the nucleotide composition of the substrate binding region. Generally, when one displacement arm is used, the substrate binding region may be 4–40 nucleotides in length,

The substrate binding region of this invention contains a "competitive binding nucleotide sequence" B that can hybridize with either the region S_B of substrate S or with the "displacement region" B' of the displaceable antisense arm A_1. The displacement region B' is a nucleotide sequence that is complementary with the competitive binding nucleotide sequence B of the substrate binding region X (and thus is

substantially similar to regions$_B$ of substrates). The length of the substrate binding region and the displacement region may vary. Generally, when one displacement arm is used, the displacement region and the competitive binding nucleotide sequence may be 2–20 nucleotides in length, or more

The displaceable antisense arm A_1 comprises the displacement region B', a first "stabilization region" C, a second stabilization region C', and optionally may contain a nonhybridizing region L_1. The first stabilization region is linked, either directly or indirectly, to the substrate binding region.

As can be seen in FIG. 1*a*, a nucleotide or sequence of nucleotides L_1 may intervene between the first and second stabilization regions.

The length of the first and second stabilization regions may vary, but generally will be within the range of 1–100 nucleotides, or, more preferably, 2–20 nucleotides.

FIG. 2 represents a hairpin ribozyme (SEQ ID NO:6) designed to cleave the 5' untranslated leader sequence of HIV (SEQ ID NO:7). The ribozyme has been modified to contain one displaceable antisense arm A_1. The substrate binding region X again contains the competitive binding nucleotide sequence B, and is linked to an exemplary first stabilization region C. The exemplary second stabilization region C', which hybridizes with the first stabilization region C, is linked to C with a short intervening nucleotide sequence L_1. Displacement region B', which is substantially identical to the S_B region of the HIV substrate S, may hybridize with the competitive binding nucleotide sequence B. The ribozyme also contains a second substrate binding region Y.

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(12) **United States Patent**
Fire et al.

(10) **Patent No.: US 6,506,559 B1**
(45) **Date of Patent: *Jan. 14, 2003**

(54) **GENETIC INHIBITION BY DOUBLE-STRANDED RNA**

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(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/215,257**

(22) Filed: **Dec. 18, 1998**

Related U.S. Application Data

(60) Provisional application No. 60/068,562, filed on Dec. 23, 1997.

(51) **Int. Cl.**[7] **C12Q 1/68**; C12N 15/85

(52) **U.S. Cl.** **435/6**; 435/91.1; 435/325

(58) **Field of Search** 514/44; 435/6, 435/91.1, 325

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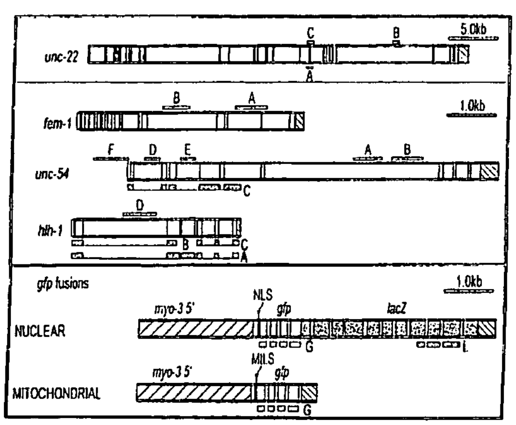
(57) **ABSTRACT**

A process is provided of introducing an RNA into a living cell to inhibit gene expression of a target gene in that cell. The process may be practiced ex vivo or in vivo. The RNA has a region with double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequences of the duplex region of the RNA and of a portion of the target gene are identical. The present invention is distinguished from prior art interference in gene expression by antisense or triple-strand methods.

22 Claims, 5 Drawing Sheets



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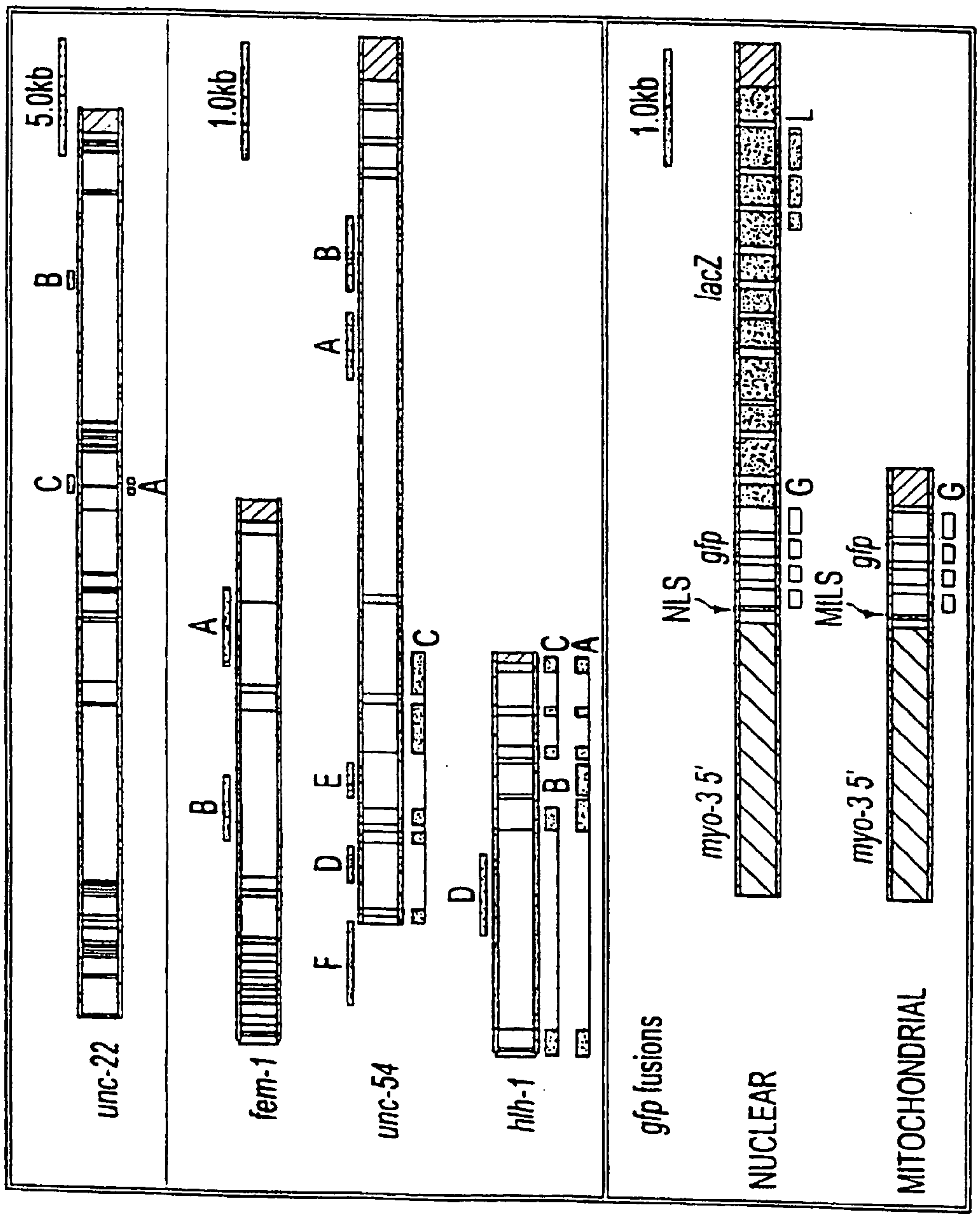


5.0kb
unc-22
1.0kb
fem-1
unc-54
hlh-1
1.0kb
gfp fusions
NUCLEAR
lacZ
NLS
gfp
myo-3 5'
MITOCHONDRIAL
MLS
gfp
myo-3 5'

FIG. 1

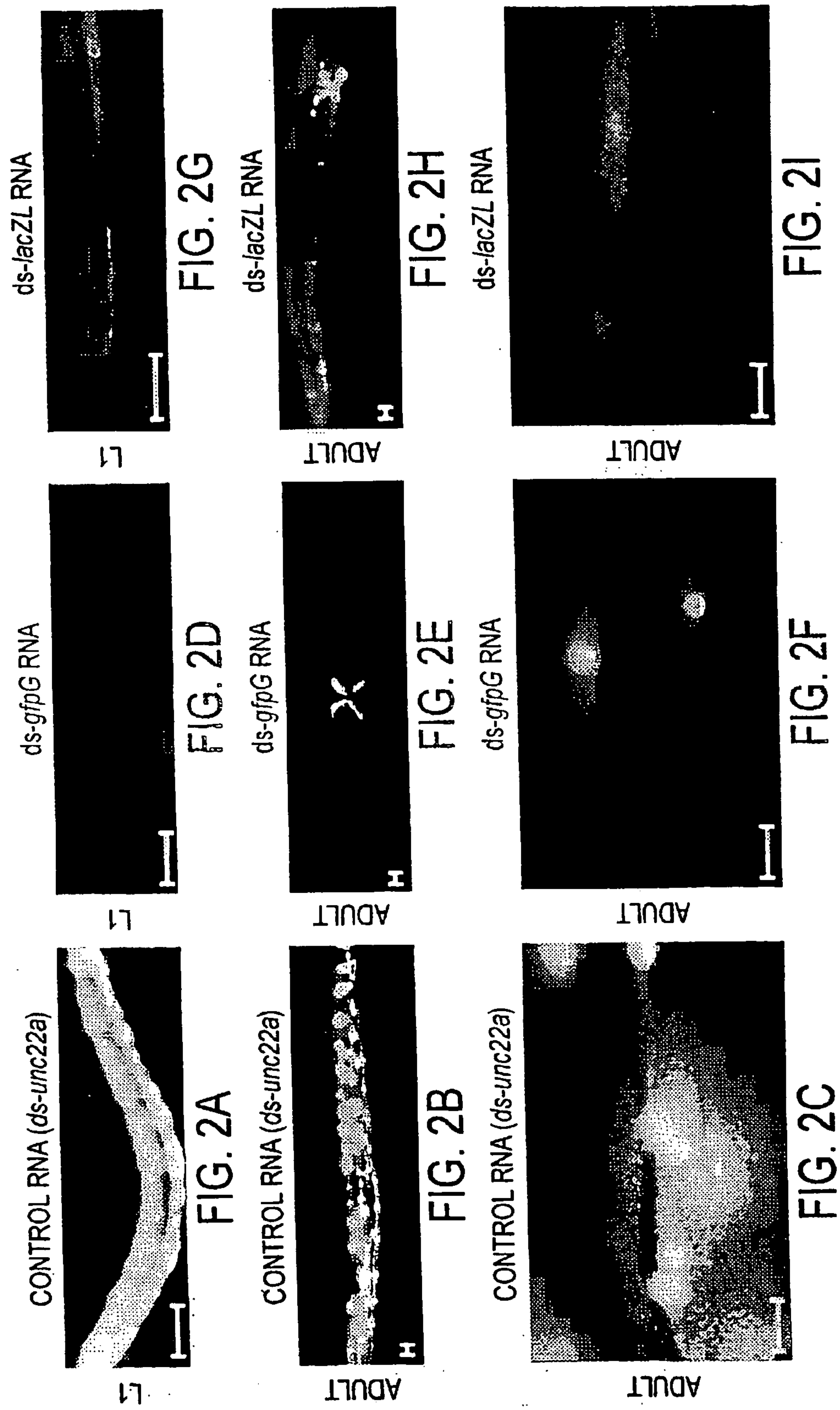


FIG. 2A FIG. 2B FIG. 2C FIG. 2D FIG. 2E FIG. 2F FIG. 2G FIG. 2H FIG. 2I

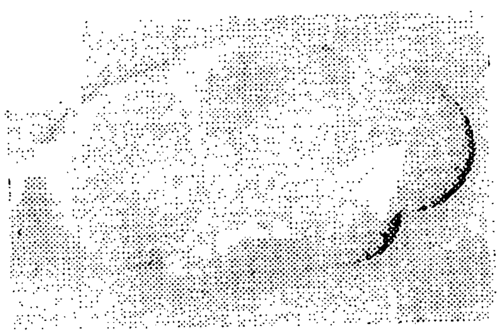


FIG. 3A


FIG. 3B

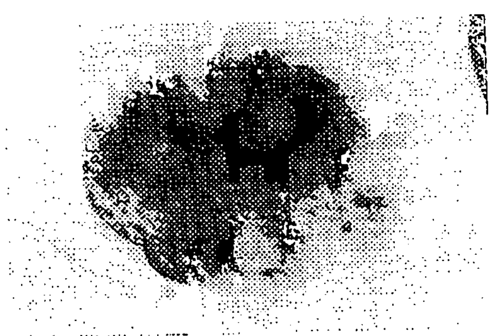


FIG. 3C

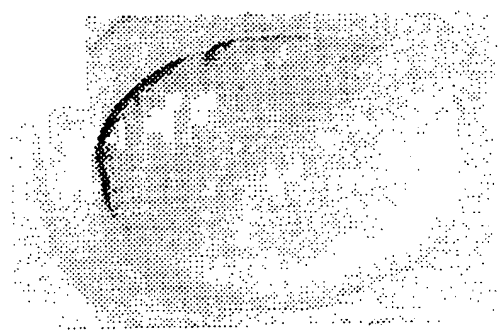
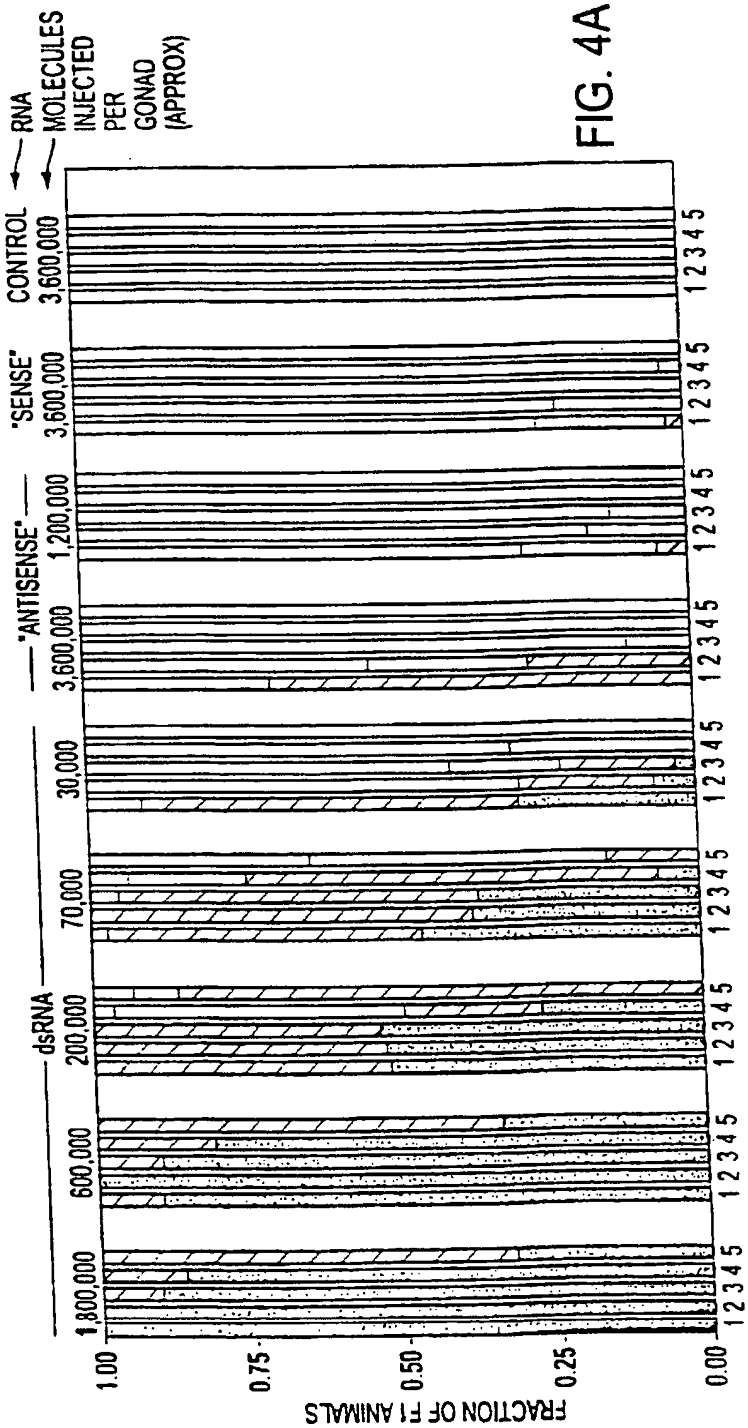
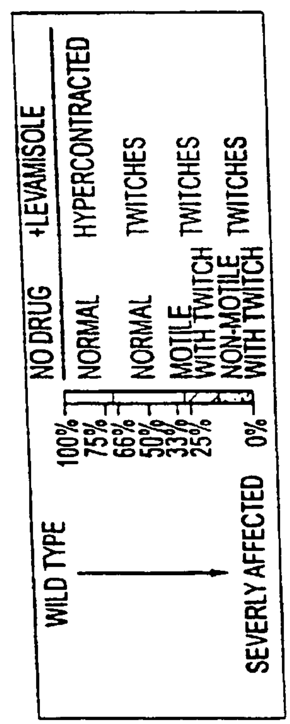


FIG. 3D



RNA
MOLECULES INJECTED PER GONAD (APPROX)
CONTROL
3,600,000
"SENSE"
3,600,000
"ANTISENSE"
1,200,000
3,600,000
dsRNA
30,000
70,000
200,000
600,000
1,800,000
1 2 3 4 5
1.00
0.75
0.50
0.25
0.00
FRACTION OF F1 ANIMALS

FIG. 4A



WILD TYPE
SEVERLY AFFECTED
NO DRUG
+LEVAMISOLE
100%
75%
66%
50%
33%
25%
0%
NORMAL
HYPERCONTRACTED
NORMAL
TWITCHES
MOTILE WITH TWITCH
TWITCHES
NON-MOTILE WITH TWITCH
TWITCHES

FIG. 4B

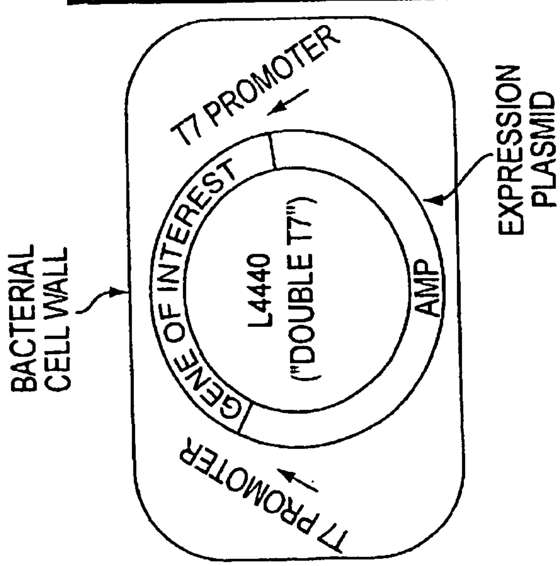


FIG. 5A

PD4251 WORMS

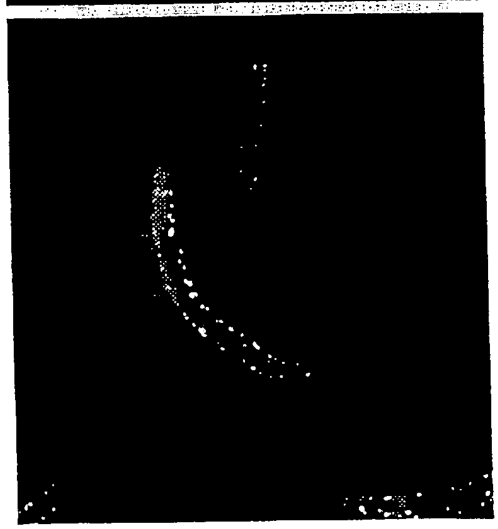


FIG. 5B

PD4251 WORMS FED BACTERIA EXPRESSING *gfp* dsRNA

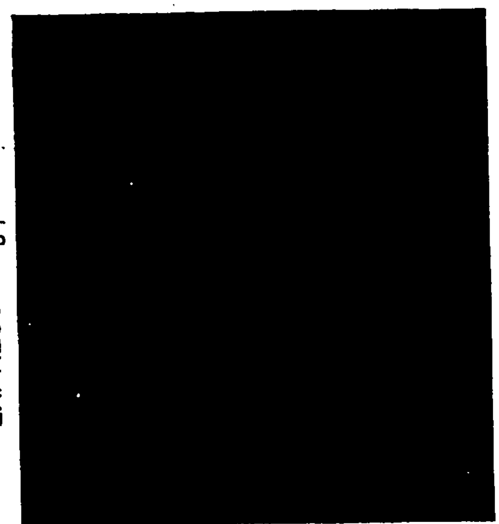


FIG. 5C

GENETIC INHIBITION BY DOUBLE-STRANDED RNA

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Appln. No. 60/068,562, filed Dec. 23, 1997. +gi

GOVERNMENT RIGHTS

This invention was made with U.S. government support under grant numbers GM-37706, GM-17164, HD-33769 and GM-07231 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to gene-specific inhibition of gene expression by double-stranded ribonucleic acid (dsRNA).

2. Description of the Related Art

Targeted inhibition of gene expression has been a long-felt need in biotechnology and genetic engineering. Although a major investment of effort has been made to achieve this goal, a more comprehensive solution to this problem was still needed.

Classical genetic techniques have been used to isolate mutant organisms with reduced expression of selected genes. Although valuable, such techniques require laborious mutagenesis and screening programs, are limited to organisms in which genetic manipulation is well established (e.g., the existence of selectable markers, the ability to control genetic segregation and sexual reproduction), and are limited to applications in which a large number of cells or organisms can be sacrificed to isolate the desired mutation. Even under these circumstances, classical genetic techniques can fail to produce mutations in specific target genes of interest, particularly when complex genetic pathways are involved. Many applications of molecular genetics require the ability to go beyond classical genetic screening techniques and efficiently produce a directed change in gene expression in a specified group of cells or organisms. Some such applications are knowledge-based projects in which it is of importance to understand what effects the loss of a specific gene product (or products) will have on the behavior of the cell or organism. Other applications are engineering based, for example: cases in which is important to produce a population of cells or organisms in which a specific gene product (or products) has been reduced or removed. A further class of applications is therapeutically based in which it would be valuable for a functioning organism (e.g., a human) to reduce or remove the amount of a specified gene product (or products). Another class of applications provides a disease model in which a physiological function in a living organism is genetically manipulated to reduce or remove a specific gene product (or products) without making a permanent change in the organism's genome.

In the last few years, advances in nucleic acid chemistry and gene transfer have inspired new approaches to engineer specific interference with gene expression. These approaches are described below.

Use of Antisense Nucleic Acids to Engineer Interference

Antisense technology has been the most commonly described approach in protocols to achieve gene-specific interference. For antisense strategies, stochiometric amounts of single-stranded nucleic acid complementary to the messenger RNA for the gene of interest are introduced into the

cell. Some difficulties with antisense-based approaches relate to delivery, stability, and dose requirements. In general, cells do not have an uptake mechanism for single-stranded nucleic acids, hence uptake of unmodified single-stranded material is extremely inefficient. While waiting for uptake into cells, the single-stranded material is subject to degradation. Because antisense interference requires that the interfering material accumulate at a relatively high concentration (at or above the concentration of endogenous mRNA), the amount required to be delivered is a major constraint on efficacy. As a consequence, much of the effort in developing antisense technology has been focused on the production of modified nucleic acids that are both stable to nuclease digestion and able to diffuse readily into cells. The use of antisense interference for gene therapy or other whole-organism applications has been limited by the large amounts of oligonucleotide that need to be synthesized from non-natural analogs, the cost of such synthesis, and the difficulty even with high doses of maintaining a sufficiently concentrated and uniform pool of interfering material in each cell.

Triple-Helix Approaches to Engineer Interference

A second, proposed method for engineered interference is based on a triple helical nucleic acid structure. This approach relies on the rare ability of certain nucleic acid populations to adopt a triple-stranded structure. Under physiological conditions, nucleic acids are virtually all single- or double-stranded, and rarely if ever form triple-stranded structures. It has been known for some time, however, that certain simple purine- or pyrimidine-rich sequences could form a triple-stranded molecule in vitro under extreme conditions of pH (i.e., in a test tube). Such structures are generally very transient under physiological conditions, so that simple delivery of unmodified nucleic acids designed to produce triple-strand structures does not yield interference. As with antisense, development of triple-strand technology for use in vivo has focused on the development of modified nucleic acids that would be more stable and more readily absorbed by cells in vivo. An additional goal in developing this technology has been to produce modified nucleic acids for which the formation of triple-stranded material proceeds effectively at physiological pH.

Co-Suppression Phenomena and Their Use in Genetic Engineering

A third approach to gene-specific interference is a set of operational procedures grouped under the name "co-suppression". This approach was first described in plants and refers to the ability of transgenes to cause silencing of an unlinked but homologous gene. More recently, phenomena similar to co-suppression have been reported in two animals: *C. elegans* and Drosophila. Co-suppression was first observed by accident, with reports coming from groups using transgenes in attempts to achieve over-expression of a potentially useful locus. In some cases the over-expression was successful while, in many others, the result was opposite from that expected. In those cases, the transgenic plants actually showed less expression of the endogenous gene. Several mechanisms have so far been proposed for transgene-mediated co-suppression in plants; all of these mechanistic proposals remain hypothetical, and no definitive mechanistic description of the process has been presented. The models that have been proposed to explain co-suppression can be placed in two different categories. In one set of proposals, a direct physical interaction at the DNA- or chromatin-level between two different chromosomal sites has been hypothesized to occur; an as-yet-unidentified mechanism would then lead to de novo methylation and subsequent suppression of gene expression.

Alternatively, some have postulated an RNA intermediate, synthesized at the transgene locus, which might then act to produce interference with the endogenous gene. The characteristics of the interfering RNA, as well as the nature of the interference process, have not been determined. Recently, a set of experiments with RNA viruses have provided some support for the possibility of RNA intermediates in the interference process. In these experiments, a replicating RNA virus is modified to include a segment from a gene of interest. This modified virus is then tested for its ability to interfere with expression of the endogenous gene. Initial results with this technique have been encouraging, however, the properties of the viral RNA that are responsible for interference effects have not been determined and, in any case, would be limited to plants which are hosts of the plant virus.

Distinction Between the Present Invention and Antisense Approaches

The present invention differs from antisense-mediated interference in both approach and effectiveness. Antisense-mediated genetic interference methods have a major challenge: delivery to the cell interior of specific single-stranded nucleic acid molecules at a concentration that is equal to or greater than the concentration of endogenous mRNA. Double-stranded RNA-mediated inhibition has advantages both in the stability of the material to be delivered and the concentration required for effective inhibition. Below, we disclose that in the model organism *C. elegans*, the present invention is at least 100-fold more effective than an equivalent antisense approach (i.e., dsRNA is at least 100-fold more effective than the injection of purified antisense RNA in reducing gene expression). These comparisons also demonstrate that inhibition by double-stranded RNA must occur by a mechanism distinct from antisense interference.

Distinction Between the Present Invention and Triple-Helix Approaches

The limited data on triple strand formation argues against the involvement of a stable triple-strand intermediate in the present invention. Triple-strand structures occur rarely, if at all, under physiological conditions and are limited to very unusual base sequence with long runs of purines and pyrimidines. By contrast, dsRNA-mediated inhibition occurs efficiently under physiological conditions, and occurs with a wide variety of inhibitory and target nucleotide sequences. The present invention has been used to inhibit expression of 18 different genes, providing phenocopies of null mutations in these genes of known function. The extreme environmental and sequence constraints on triple-helix formation make it unlikely that dsRNA-mediated inhibition in *C. elegans* is mediated by a triple-strand structure.

Distinction Between Present Invention and Co-Suppression Approaches

The transgene-mediated genetic interference phenomenon called co-suppression may include a wide variety of different processes. From the viewpoint of application to other types of organisms, the co-suppression phenomenon in plants is difficult to extend. A confounding aspect in creating a general technique based on co-suppression is that some transgenes in plants lead to suppression of the endogenous locus and some do not. Results in *C. elegans* and Drosophila indicate that certain transgenes can cause interference (i.e., a quantitative decrease in the activity of the corresponding endogenous locus) but that most transgenes do not produce such an effect. The lack of a predictable effect in plants, nematodes, and insects greatly limits the usefulness of simply adding transgenes to the genome to interfere with gene expression. Viral-mediated co-suppression in plants appears to be quite effective, but has a number of drawbacks. First, it is not clear what aspects of the viral structure are critical for the observed interference. Extension to another system would require discovery of a virus in that system which would have these properties, and such a library of useful viral agents are not available for many organisms. Second, the use of a replicating virus within an organism to effect genetic changes (e.g., long- or short-term gene therapy) requires considerably more monitoring and oversight for deleterious effects than the use of a defined nucleic acid as in the present invention.

The present invention avoids the disadvantages of the previously-described methods for genetic interference. Several advantages of the present invention are discussed below, but numerous others will be apparent to one of ordinary skill in the biotechnology and genetic engineering arts.

SUMMARY OF THE INVENTION

A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

The RNA may comprise one or more strands of polymerized ribonucleotide; it may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. RNA containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). RNA may be synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For

transcription from a transgene in vivo or an expression construct, a regulatory region may be used to transcribe the RNA strand (or strands).

The RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

The advantages of the present invention include: the ease of introducing double-stranded RNA into cells, the low concentration of RNA which can be used, the stability of double-stranded RNA, and the effectiveness of the inhibition. The ability to use a low concentration of a naturally-occurring nucleic acid avoids several disadvantages of anti-sense interference. This invention is not limited to in vitro use or to specific sequence compositions, as are techniques based on triple-strand formation. And unlike antisense interference, triple-strand interference, and co-suppression, this invention does not suffer from being limited to a particular set of target genes, a particular portion of the target gene's nucleotide sequence, or a particular transgene or viral delivery method. These concerns have been a serious obstacle to designing general strategies according to the prior art for inhibiting gene expression of a target gene of interest.

Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; unc-22[9], unc-54[12], fem-1[14], and hlh-1[15]).

FIGS. 2A–I show analysis of inhibitory RNA effects in individual cells. These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins, nuclear GFP-LacZ and mitochondrial GFP. The micrographs show progeny of injected animals visualized by a fluorescence microscope. Panels A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (ds-unc22A). Panels D–F show progeny of animals injected with ds-gfpG. Panels G–I demonstrate specificity. Animals are injected with ds-lacZL RNA, which should affect the nuclear but not the mitochondrial reporter construct. Panel H shows a typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars are 20 μm.

FIGS. 3A–D show effects of double-stranded RNA corresponding to mex-3 on levels of the endogenous mRNA. Micrographs show in situ hybridization to embryos (dark stain). Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous mex-3 RNA[20]). Panel C: Embryo from a parent injected with purified mex-3B antisense RNA. These embryos and the parent animals retain the mex-3 mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with dsRNA corresponding to mex-3B; no mex-3 RNA was detected. Scale: each embryo is approximately 50 μm in length.

FIG. 4 shows inhibitory activity of unc-22A as a function of structure and concentration. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included. Progeny cohort groups are labeled 1 for 0–6 hours, 2 for 6–15 hours, 3 for 15–27 hours, 4 for 27–41 hours, and 5 for 41–56 hours The bottom-left diagram shows genetically derived relationship between unc-22 gene dosage and behavior based on analyses of unc-22 heterozygotes and polyploids[8,3].

FIGS. 5A–C show examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. Panel A: General strategy for production of dsRNA by cloning a segment of interest between flanking copies of the bacteriophage T7 promoter and transcribing both strands of the segment by transfecting a bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter. Panel B: A GFP-expressing *C. elegans* strain, PD4251 (see FIG. 2), fed on a native bacterial host. Panel C: PD4251 animals reared on a diet of bacteria expressing dsRNA corresponding to the coding region for gfp.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing sequence-specific inhibition of gene expression by introducing double-stranded RNA (dsRNA). A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell. Inhibition is sequence-specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell (i.e., a cellular gene), an endogenous gene (i.e., a cellular gene present in the genome), a transgene (i.e., a gene construct inserted at an ectopic site in the genome of the cell), or a gene from a pathogen which is capable of infecting an organism from which the cell is derived. Depending on the particular target gene and the dose of double stranded RNA material delivered, this process may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole

organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

The RNA may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by in vitro enzymatic or organic synthesis.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

RNA containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50° C. or 70° C. hybridization for 12–16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases.

As disclosed herein, 100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis; field crops (e.g., alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative generae of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tfhchonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphalenchus, Criconemella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, and Xiphinema). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or nondividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

RNA may be synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene in vivo or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and

acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art[32,33,34] (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by in vitro enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. For example, the RNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. We disclose herein that in *C. elegans*, double-stranded RNA introduced outside the cell inhibits gene expression. Vascular or extravascular circulation, the blood or lymph system, the phloem, the roots, and the cerebrospinal fluid are sites where the RNA may be introduced. A transgenic organism that expresses RNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.

Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

The present invention may be used to introduce RNA into a cell for the treatment or prevention of disease. For example, dsRNA may be introduced into a cancerous cell or tumor and thereby inhibit gene expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene may be selected which is required for initiation or maintenance of the disease/pathology. Treatment would include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

A gene derived from any pathogen may be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. The inhibitory RNA could be introduced in cells in vitro or ex vivo and then subsequently placed into an animal to affect therapy, or directly treated by in vivo administration. A method of gene therapy can be envisioned. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, can be envisioned.

The present invention could be used for treatment or development of treatments for cancers of any type, including solid tumors and leukemias, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (e.g., B cell, mixed cell, null cell, T cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast cell, and myeloid), histiocytosis malignant, Hodgkin disease, immunoproliferative small, non-Hodgkin lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, craniopharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adenocarcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, Leydig cell tumor, papilloma, Sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (e.g., Ewing, experimental, Kaposi, and mast cell), neoplasms (e.g., bone, breast, diges-

tive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia, and for treatment of other conditions in which cells have become immortalized or transformed. The invention could be used in combination with other treatment modalities, such as chemotherapy, cryotherapy, hyperthermia, radiation therapy, and the like.

As disclosed herein, the present invention may is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, TP53, and WT1); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

The present invention could comprise a method for producing plants with reduced susceptibility to climatic injury, susceptibility to insect damage, susceptibility to infection by a pathogen, or altered fruit ripening characteristics. The targeted gene may be an enzyme, a plant structural protein, a gene involved in pathogenesis, or an enzyme that is involved in the production of a non-proteinaceous part of the plant (i.e., a carbohydrate or lipid). If an expression construct is used to transcribe the RNA in a plant, transcription by a wound- or stress-inducible; tissue-specific (e.g., fruit, seed, anther, flower, leaf, root); or otherwise regulatable (e.g., infection, light, temperature, chemical) promoter may be used. By inhibiting enzymes at one or more points in a metabolic pathway or genes involved in pathogenesis, the effect may be enhanced: each activity will be affected and the effects may be magnified by targeting multiple different components. Metabolism may also be manipulated by inhibiting feedback control in the pathway or production of unwanted metabolic byproducts.

The present invention may be used to reduce crop destruction by other plant pathogens such as arachnids, insects, nematodes, protozoans, bacteria, or fungi. Some such plants and their pathogens are listed in Index of plant Diseases in the United States (U.S. Dept. of Agriculture Handbook No. 165, 1960); Distribution of Plant-Parasitic Nematode Species in North America (Society of Nematologists, 1985); and Fungi on Plants and Plant Products in the United States (American Phytopathological Society, 1989). Insects with reduced ability to damage crops or improved ability to prevent other destructive insects from damaging crops may be produced. Furthermore, some nematodes are vectors of plant pathogens, and may be attacked by other beneficial nematodes which have no effect on plants. Inhibition of target gene activity could be used to delay or prevent entry into a particular developmental step (e.g., metamorphosis), if plant disease was associated with a particular stage of the pathogen's life cycle. Interactions between pathogens may also be modified by the invention to limit crop damage. For example, the ability of beneficial nematodes to attack their harmful prey may be enhanced by inhibition of behavior-controlling nematode genes according to the invention.

Although pathogens cause disease, some of the microbes interact with their plant host in a beneficial manner. For example, some bacteria are involved in symbiotic relationships that fix nitrogen and some fungi produce phytohormones. Such beneficial interactions may be promoted by using the present invention to inhibit target gene activity in the plant and/or the microbe.

Another utility of the present invention could be a method of identifying gene function in an organism comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceutics, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for the yeast, *D. melanogaster*, and *C. elegans* genomes, can be coupled with the invention to determine gene function in an organism (e.g., nematode). The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which RNA can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell/organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96-well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process. Solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The amplified RNA can be fed directly to, injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by in vivo or in vitro transcription from an expression construct used to produce the library. The construct can be replicated as individual clones

of the library and transcribed to produce the RNA; each clone can then be fed to, or injected into, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example: arabidopsis, bacteria, drosophila, fungi, nematodes, viruses, zebrafish, and tissue culture cells derived from mammals.

A nematode or other organism that produces a colorimetric, fluorogenic, or luminescent signal in response to a regulated promoter (e.g., transfected with a reporter gene construct) can be assayed in an HTS format to identify DNA-binding proteins that regulate the promoter. In the assay's simplest form, inhibition of a negative regulator results in an increase of the signal and inhibition of a positive regulator results in a decrease of the signal.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the in vitro or in vivo introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Pesticides may include the RNA molecule itself, an expression construct capable of expressing the RNA, or organisms transfected with the expression construct. The pesticide of the present invention may serve as an arachnicide, insecticide, nematicide, viricide, bactericide, and/or fungicide. For example, plant parts that are accessible above ground (e.g., flowers, fruits, buds, leaves, seeds, shoots, bark, stems) may be sprayed with pesticide, the soil may be soaked with pesticide to access plant parts growing beneath ground level, or the pest may be contacted with pesticide directly. If pests interact with each other, the RNA may be transmitted between them. Alternatively, if inhibition of the target gene results in a beneficial effect on plant growth or development, the aforementioned RNA, expression construct, or transfected organism may be considered a nutritional agent. In either case, genetic engineering of the plant is not required to achieve the objectives of the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

Used as either an pesticide or nutrient, a formulation of the present invention may be delivered to the end user in dry or liquid form: for example, as a dust, granulate, emulsion, paste, solution, concentrate, suspension, or encapsulation. Instructions for safe and effective use may also be provided with the formulation. The formulation might be used directly, but concentrates would require dilution by mixing with an extender provided by the formulator or the end user. Similarly, an emulsion, paste, or suspension may require the end user to perform certain preparation steps before application. The formulation may include a combination of chemical additives known in the art such as solid carriers, minerals, solvents, dispersants, surfactants, emulsifiers, tackifiers, binders, and other adjuvants. Preservatives and stabilizers may also be added to the formulation to facilitate storage. The crop area or plant may also be treated simultaneously or separately with other pesticides or fertilizers. Methods of application include dusting, scattering or pouring, soaking, spraying, atomizing, and coating. The precise physical form and chemical composition of the formulation, and its method of application, would be chosen to promote the objectives of the invention and in accordance with prevailing circumstances. Expression constructs and transfected hosts capable of replication may also promote the persistence and/or spread of the formulation.

Description of the dsRNA Inhibition Phenomenon in *C. elegans*

The operation of the present invention was shown in the model genetic organism *Caenorhabditis elegans*.

Introduction of RNA into cells had been seen in certain biological systems to interfere with function of an endogenous gene [1,2]. Many such effects were believed to result from a simple antisense mechanism dependent on hybridization between injected single-stranded RNA and endogenous transcripts. In other cases, a more complex mechanism had been suggested. One instance of an RNA-mediated mechanism was RNA interference (RNAi) phenomenon in the nematode *C. elegans*. RNAi had been used in a variety of studies to manipulate gene expression[3,4].

Despite the usefulness of RNAi in C. elegans, many features had been difficult to explain. Also, the lack of a clear understanding of the critical requirements for interfering RNA led to a sporadic record of failure and partial success in attempts to extend RNAi beyond the earliest stages following injection. A statement frequently made in the literature was that sense and antisense RNA preparations are each sufficient to cause interference[3,4]. The only precedent for such a situation was in plants where the process of cosuppression had a similar history of usefulness in certain cases, failure in others, and no ability to design interference protocols with a high chance of success. Working with *C. elegans*, we discovered an RNA structure that would give

effective and uniform genetic inhibition. The prior art did not teach or suggest that RNA structure was a critical feature for inhibition of gene expression. Indeed the ability of crude sense and antisense preparations to produce interference[3,4] had been taken as an indication that RNA structure was not a critical factor. Instead, the extensive plant literature and much of the ongoing research in *C. elegans* was focused on the possibility that detailed features of the target gene sequence or its chromosomal locale was the critical feature for interfering with gene expression.

The inventors carefully purified sense or antisense RNA for unc-22 and tested each for gene-specific inhibition. While the crude sense and antisense preparations had strong interfering activity, it was found that the purified sense and antisense RNAs had only marginal inhibitory activity. This was unexpected because many techniques in molecular biology are based on the assumption that RNA produced with specific in vitro promoters (e.g., T3 or T7 RNA polymerase), or with characterized promoters in vivo, is produced predominantly from a single strand. The inventors had carried out purification of these crude preparations to investigate whether a small fraction of the RNA had an unusual structure which might be responsible for the observed genetic inhibition. To rigorously test whether double-stranded character might contribute to genetic inhibition, the inventors carried out additional purification of single-stranded RNAs and compared inhibitory activities of individual strands with that of the double-stranded hybrid.

The following examples are meant to be illustrative of the present invention; however, the practice of the invention is not limited or restricted in any way by them.

Analysis of RNA-Mediated Inhibition of *C. elegans* Genes

The unc-22 gene was chosen for initial comparisons of activity as a result of previous genetic analysis that yields a semi-quantitative comparison between unc-22 gene activity and the movement phenotypes of animals[3,8]: decreases in activity produce an increasingly severe twitching phenotype, while complete loss of function results in the additional appearance of muscle structural defects and impaired motility. unc-22 encodes an abundant but non-essential myofilament protein[7-9]. unc-22 mRNA is present at several thousand copies per striated muscle cell[3].

Purified antisense and sense RNAs covering a 742 nt segment of unc-22 had only marginal inhibitory activity, requiring a very high dose of injected RNA for any observable effect (FIG. 4). By contrast, a sense+antisense mixture produced a highly effective inhibition of endogenous gene activity (FIG. 4). The mixture was at least two orders of magnitude more effective than either single strand in inhibiting gene expression. The lowest dose of the sense+antisense mixture tested, approximately 60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. unc-22 expression begins in embryos with approximately 500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent inhibitory activity of the sense+antisense mixture could reflect formation of double-stranded RNA (dsRNA), or conceivably some alternate synergy between the strands. Electrophoretic analysis indicated that the injected material was predominantly double stranded. The dsRNA was gel purified from the annealed mixture and found to retain potent inhibitory activity. Although annealing prior to injection was compatible with inhibition, it was not necessary. Mixing of sense and antisense RNAs in low salt (under conditions of minimal dsRNA formation), or rapid sequential injection of sense and antisense strands, were sufficient to allow complete inhibition. A long interval (>1 hour) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in inhibitory activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the complementary strand.

An issue of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic response mechanism[10]. Conceivably, the inventive sense+antisense synergy could reflect a non-specific potentiation of antisense effects by such a panic mechanism. This was not found to be the case: co-injection of dsRNA segments unrelated to unc-22 did not potentiate the ability of unc-22 single strands to mediate inhibition. Also investigated was whether double-stranded structure could potentiate inhibitory activity when placed in cis to a single-stranded segment. No such potentiation was seen; unrelated double-stranded sequences located 5' or 3' of a single-stranded unc-22 segment did not stimulate inhibition. Thus potentiation of gene-specific inhibition was observed only when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by unc-22 dsRNA was specific. Progeny of injected animals exhibited behavior indistinguishable from characteristic unc-22 loss of function mutants. Target-specificity of dsRNA effects using three additional genes with well characterized phenotypes (FIG. 1 and Table 1). unc-54 encodes a body wall muscle myosin heavy chain isoform required for full muscle contraction[7,11,12], fem-1 encodes an ankyrin-repeat containing protein required in hermaphrodites for sperm production[13,14], and hlh-1 encodes a *C. elegans* homolog of the myoD family required for proper body shape and motility[15,16]. For each of these genes, injection of dsRNA produced progeny broods exhibiting the known null mutant phenotype, while the purified single strands produced no significant reduction in gene expression. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from inhibition of the corresponding gene. The exception (segment unc54C, which led to an embryonic and larval arrest phenotype not seen with unc-54 null mutants) was illustrative. This segment covers the highly conserved myosin motor domain, and might have been expected to inhibit the activity of other highly related myosin heavy chain genes[17]. This interpretation would support uses of the present invention in which nucleotide sequence comparison of dsRNA and target gene show less than 100% identity. The unc54C segment has been unique in our overall experience to date: effects of 18 other dsRNA segments have all been limited to those expected from characterized null mutants.

The strong phenotypes seen following dsRNA injection are indicative of inhibitory effects occurring in a high fraction of cells. The unc-54 and hlh-1 muscle phenotypes, in particular, are known to result from a large number of defective muscle cells[11,16]. To examine inhibitory effects of dsRNA on a cellular level, a transgenic line expressing two different GFP-derived fluorescent reporter proteins in body muscle was used. Injection of dsRNA directed to gfp produced dramatic decreases in the fraction of fluorescent cells (FIG. 2). Both reporter proteins were absent from the negative cells, while the few positive cells generally expressed both GFP forms.

The pattern of mosaicism observed with gfp inhibition was not random. At low doses of dsRNA, the inventors saw frequent inhibition in the embryonically-derived muscle cells present when the animal hatched. The inhibitory effect in these differentiated cells persisted through larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically-derived

striated muscles are born during early larval stages and were more resistant to inhibition. These cells have come through additional divisions (13–14 versus 8–9 for embryonic muscles[18,19]). At high concentrations of gfp dsRNA, inhibition was noted in virtually all striated bodywall muscles, with occasional single escaping cells including cells born in embryonic or postembryonic stages. The nonstriated vulval muscles, born during late larval development, appeared resistant to genetic inhibition at all tested concentrations of injected RNA. The latter result is important for evaluating the use of the present invention in other systems. First, it indicates that failure in one set of cells from an organism does not necessarily indicate complete non-applicability of the invention to that organism. Second, it is important to realize that not all tissues in the organism need to be affected for the invention to be used in an organism. This may serve as an advantage in some situations.

A few observations serve to clarify the nature of possible targets and mechanisms for RNA-mediated genetic inhibition in *C. elegans:*

First, dsRNA segments corresponding to a variety of intron and promoter sequences did not produce detectable inhibition (Table 1). Although consistent with possible inhibition at a post-transcriptional level, these experiments do not rule out inhibition at the level of the gene.

Second, dsRNA injection produced a dramatic decrease in the level of the endogenous mRNA transcript (FIG. 3). Here, a mex-3 transcript that is abundant in the gonad and early embryos[20] was targeted, where straightforward in situ hybridization can be performed. No endogenous mex-3 mRNA was observed in animals injected with a dsRNA segment derived from mex-3 (FIG. 3D), but injection of purified mex-3 antisense RNA resulted in animals that retained substantial endogenous mRNA levels (FIG. 3C).

Third, dsRNA-mediated inhibition showed a surprising ability to cross cellular boundaries. Injection of dsRNA for unc-22, gfp, or lacZ into the body cavity of the head or tail produced a specific and robust inhibition of gene expression in the progeny brood (Table 2). Inhibition was seen in the progeny of both gonad arms, ruling out a transient "nicking" of the gonad in these injections. dsRNA injected into body cavity or gonad of young adults also produced gene-specific inhibition in somatic tissues of the injected animal (Table 2).

Table 3 shows that *C. elegans* can respond in a gene-specific manner to dsRNA encountered in the environment. Bacteria are a natural food source for *C. elegans*. The bacteria are ingested, ground in the animal's pharynx, and the bacterial contents taken up in the gut. The results show that *E. coli* bacteria expressing dsRNAs can confer specific inhibitory effects on *C. elegans* nematode larvae that feed on them.

Three *C. elegans* genes were analyzed. For each gene, corresponding dsRNA was expressed in *E. coli* by inserting a segment of the coding region into a plasmid construct designed for bidirectional transcription by bacteriophage T7 RNA polymerase. The dsRNA segments used for these experiments were the same as those used in previous microinjection experiments (see FIG. 1). The effects resulting from feeding these bacteria to *C. elegans* were compared to the effects achieved by microinjecting animals with dsRNA.

The *C. elegans* gene unc-22 encodes an abundant muscle filament protein. unc-22 null mutations produce a characteristic and uniform twitching phenotype in which the animals can sustain only transient muscle contraction. When wild-type animals were fed bacteria expressing a dsRNA segment from unc-22, a high fraction (85%) exhibited a weak but still distinct twitching phenotype characteristic of partial loss of function for the unc-22 gene. The *C. elegans* fem-1 gene encodes a late component of the sex determination pathway. Null mutations prevent the production of sperm and lead euploid (XX) animals to develop as females, while wild type XX animals develop as hermaphrodites. When wild-type animals were fed bacteria expressing dsRNA corresponding to fem-1, a fraction (43%) exhibit a sperm-less (female) phenotype and were sterile. Finally, the ability to inhibit gene expression of a transgene target was assessed. When animals carrying a gfp transgene were fed bacteria expressing dsRNA corresponding to the gfp reporter, an obvious decrease in the overall level of GFP fluorescence was observed, again in approximately 12% of the population (see FIG. 5, panels B and C).

The effects of these ingested RNAs were specific. Bacteria carrying different dsRNAs from fem-1 and gfp produced no twitching, dsRNAs from unc-22 and fem-1 did not reduce gfp expression, and dsRNAs from gfp and unc-22 did not produce females. These inhibitory effects were apparently mediated by dsRNA: bacteria expressing only the sense or antisense strand for either gfp or unc-22 caused no evident phenotypic effects on their *C. elegans* predators.

Table 4 shows the effects of bathing *C. elegans* in a solution containing dsRNA. Larvae were bathed for 24 hours in solutions of the indicated dsRNAs (1 mg/ml), then allowed to recover in normal media and allowed to grow under standard conditions for two days. The unc-22 dsRNA was segment ds-unc22A from FIG. 1. pos-1 and sqt-3 dsRNAs were from the full length cDNA clones. pos-1 encodes an essential maternally provided component required early in embyogenesis. Mutations removing pos-1 activity have an early embryonic arrest characteristic of skn-like mutations[29,30]. Cloning and activity patterns for sqt-3 have been described[31]. *C. elegans* sqt-3 mutants have mutations in the col-1 collagen gene[31]. Phenotypes of affected animals are noted. Incidences of clear phenotypic effects in these experiments were 5–10% for unc-22, 50% for pos-1, and 5% for sqt-3. These are frequencies of unambiguous phenocopies; other treated animals may have had marginal defects corresponding to the target gene that were not observable. Each treatment was fully gene-specific in that unc-22 dsRNA produced only Unc-22 phenotypes, pos-1 dsRNA produced only Pos-1 phenotypes, and sqt-3 dsRNA produced only Sqt-3 phenotypes.

Some of the results described herein were published after the filing of our provisional application. Those publications and a review can be cited as Fire, A., et al. Nature, 391, 806–811, 1998; Timmons, L. & Fire, A. Nature, 395, 854, 1998; and Montgomery, M. K. & Fire, A. Trends in Genetics, 14, 255–258, 1998.

The effects described herein significantly augment available tools for studying gene function in *C. elegans* and other organisms. In particular, functional analysis should now be possible for a large number of interesting coding regions[21] for which no specific function have been defined. Several of these observations show the properties of dsRNA that may affect the design of processes for inhibition of gene expression. For example, one case was observed in which a nucleotide sequence shared between several myosin genes may inhibit gene expression of several members of a related gene family.

Methods of RNA Synthesis and Microinjection

RNA was synthesized from phagemid clones with T3 and T7 RNA polymerase[6], followed by template removal with two sequential DNase treatments. In cases where sense, antisense, and mixed RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original "sense" and "antisense" preparations. Nonetheless,

RNA species accounting for less than 10% of purified RNA preparations would not have been observed. Without gel purification, the "sense" and "antisense" preparations produced significant inhibition. This inhibitory activity was reduced or eliminated upon gel purification. By contrast, sense+antisense mixtures of gel purified and non-gel-purified RNA preparations produced identical effects.

Following a short (5 minute) treatment at 68° C. to remove secondary structure, sense+antisense annealing was carried out in injection buffer[27] at 37° C. for 10–30 minutes. Formation of predominantly double stranded material was confirmed by testing migration on a standard (non-denaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for double-stranded RNA of the appropriate length. Co-incubation of the two strands in a low-salt buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of double-stranded RNA in vitro. Non-annealed sense+antisense RNAs for unc22B and gfpG were tested for inhibitory effect and found to be much more active than the individual single strands, but 2–4 fold less active than equivalent pre-annealed preparations.

After pre-annealing of the single strands for unc22A, the single electrophoretic species corresponding in size to that expected for dsRNA was purified using two rounds of gel electrophoresis. This material retained a high degree of inhibitory activity.

Except where noted, injection mixes were constructed so animals would receive an average of 0.5×10^6 to 1.0×10^6 molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections were compared with equal masses of RNA (i.e., dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible; however, such variability would not affect any of the conclusions drawn herein.

Methods for Analysis of Phenotypes

Inhibition of endogenous genes was generally assayed in a wild type genetic background (N2). Features analyzed included movement, feeding, hatching, body shape, sexual identity, and fertility. Inhibition with gfp[27] and lacZ activity was assessed using strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccIs4251) made up of three plasmids: pSAK4 (myo-3 promoter driving mitochondrially targeted GFP), pSAK2 (myo-3 promoter driving a nuclear targeted GFP-LacZ fusion), and a dpy-20 subclone[26] as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing a facile distinction between cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was performed by inserting the microinjection needle into the gonadal syncitium of adults and expelling 20–100 pl of solution (see Reference 25). Body cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells was another effective means of RNA delivery, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16 hour intervals. This yields a series of semi-synchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short "clearance" interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. After the clearance period, individuals are produced which show the inhibitory phenotype. After injected animals have produced eggs for several days, gonads can in some cases "revert" to produce incompletely affected or phenotypically normal progeny.

Additional Description of the Results

FIG. 1 shows genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; sequence references are as follows: unc-22[9], unc-54[12], fem-1[14], and hlh-1[15]). These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for inhibitory effects is designated with the name of the gene followed by a single letter (e.g., unc22C). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.

The effects of inhibitory RNA were analyzed in individual cells (FIG. 2, panels A–H). These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene. ds-unc22A RNA was injected as a negative control. GFP expression in progeny of these injected animals was not affected. The GFP patterns of these progeny appeared identical to the parent strain, with prominent fluorescence in nuclei (the nuclear localized GFP-LacZ) and mitochondria (the mitochondrially targeted GFP): young larva (FIG. 2A), adult (FIG. 2B), and adult body wall at high magnification (FIG. 2C).

In contrast, the progeny of animals injected with ds-gfpG RNA are affected (FIGS. 2D–F). Observable GFP fluorescence is completely absent in over 95% of the cells. Few active cells were seen in larvae (FIG. 2D shows a larva with one active cell; uninjected controls show GFP activity in all 81 body wall muscle cells). Inhibition was not effective in all tissues: the entire vulval musculature expressed active GFP in an adult animal (FIG. 2E). Rare GFP positive body wall muscle cells were also seen adult animals (two active cells are shown in FIG. 2F). Inhibition was target specific (FIGS. 2G–I). Animals were injected with ds-lacZL RNA, which should affect the nuclear but not the mitochondrial reporter construct. In the animals derived from this injection, mitochondrial-targeted GFP appeared unaffected while the nuclear-targeted GFP-LacZ was absent from almost all cells (larva in FIG. 2G). A typical adult lacked nuclear GFP-LacZ in almost all body-wall muscles but retained activity in vulval muscles (FIG. 2H). Scale bars in FIG. 2 are 20 μm.

The effects of double-stranded RNA corresponding to mex-3 on levels of the endogenous mRNA was shown by in situ hybridization to embryos (FIG. 3, panels A–D). The 1262 nt mex-3 cDNA clone[20] was divided into two segments, mex-3A and mex3B with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. mex-3B antisense or dsRNA was injected into the gonads of adult animals, which were maintained under standard culture conditions for 24 hours before fixation and in situ hybridization (see

Reference 5). The mex-3B dsRNA produced 100% embryonic arrest, while >90% of embryos from the antisense injections hatched. Antisense probes corresponding to mex-3A were used to assay distribution of the endogenous mex-3 mRNA (dark stain). Four-cell stage embryos were assayed; similar results were observed from the 1 to 8 cell stage and in the germlime of injected adults. The negative control (the absence of hybridization probe) showed a lack of staining (FIG. 3A). Embryos from uninjected parents showed a normal pattern of endogenous mex-3 RNA (FIG. 3B). The observed pattern of mex-3 RNA was as previously described in Reference 20. Injection of purified mex-3B antisense RNA produced at most a modest effect: the resulting embryos retained mex-3 mRNA, although levels may have been somewhat less than wild type (FIG. 3C). In contrast, no mex-3 RNA was detected in embryos from parents injected with dsRNA corresponding to mex-3 (FIG. 3D). The scale of FIG. 3 is such that each embryo is approximately 50 μm in length.

Gene-specific inhibitory activity by unc-22A RNA was measured as a function of RNA structure and concentration (FIG. 4). Purified antisense and sense RNA from unc22A were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (gfpG). Injected animals were transferred to fresh culture plates 6 hours (columns labeled 1), 15 hours (columns labeled 2), 27 hours (columns labeled 3), 41 hours (columns labeled 4), and 56 hours (columns labeled 5) after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included in the graph. The bottom-left diagram shows the genetically derived relationship between unc-22 gene dosage and behavior based on analyses of unc-22 heterozygotes and polyploids[8, 3].

FIGS. 5A–C show a process and examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. A general strategy for production of dsRNA is to clone segments of interest between flanking copies of the bacteriophage T7 promoter into a bacterial plasmid construct (FIG. 5A). A bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter was used as a host. A nuclease-resistant dsRNA was detected in lysates of transfected bacteria. Comparable inhibition results were obtained with the two bacterial expression systems. A GFP-expressing *C. elegans* strain, PD4251 (see FIG. 2), was fed on a native bacterial host. These animals show a uniformly high level of GFP fluorescence in body muscles (FIG. 5B). PD4251 animals were also reared on a diet of bacteria expressing dsRNA corresponding to the coding region for gfp. Under the conditions of this experiment, 12% of these animals showed dramatic decreases in GFP (FIG. 5C). As an alternative strategy, single copies of the T7 promoter were used to drive expression of an inverted-duplication for a segment of the target gene, either unc-22 or gfp. This was comparably effective.

All references (e.g., books, articles, applications, and patents) cited in this specification are indicative of the level of skill in the art and their disclosures are incorporated herein in their entirety.

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TABLE 1

Effects of sense, antisense, and mixed RNAs on progeny of injected animals.

Gene and Segment		Size	Injected RNA	F1 Phenotype
unc-22				unc-22 null mutants: strong twitchers[7,8]
unc22A[a]	exon 21–22	742	sense	wild type
			antisense	wild type
			sense + antisense	strong twitchers (100%)
unc22B	exon27	1033	sense	wild type
			antisense	wild type

TABLE 1-continued

Effects of sense, antisense, and mixed RNAs on progeny of injected animals.

Gene and Segment		Size	Injected RNA	F1 Phenotype
			sense + antisense	strong twitchers (100%)
unc22C	exon 21–22[b]	785	sense + antisense	strong twitchers (100%)
fem-1				fem-1 null mutants: female (no sperm)[13]
fem1A	exon 10[c]	531	sense	hermaphrodite (98%)
			antisense	hermaphrodite (>98%)
			sense + antisense	female (72%)
fem1B	intron 8	556	sense + antisense	hermaphrodite (>98%)
unc-54				unc-54 null mutants: paralyzed[7,11]
unc54A	exon 6	576	sense	wild type (100%)
			antisense	wild type (100%)
			sense + antisense	paralyzed (100%)[d]
unc54B	exon 6	651	sense	wild type (100%)
			antisense	wild type (100%)
			sense + antisense	paralyzed (100%)[d]
unc54C	exon 1–5	1015	sense + antisense	arrested embryos and larvae (100%)
unc54D	promoter	567	sense + antisense	wild type (100%)
unc54E	intron 1	369	sense + antisense	wild type (100%)
unc54F	intron 3	386	sense + antisense	wild type (100%)
hlh-1				hlh-1 null mutants: lumpy-dumpy larvae[16]
h/h1A	exons 1–6	1033	sense	wild type (<2% lpy-dpy)
			antisense	wild type (<2% lpy-dpy)
			sense + antisense	lpy-dpy larvae (>90%)[e]
hlh1B	exons 1–2	438	sense + antisense	lpy-dpy larvae (>80%)[e]
hlh1C	exons 4–6	299	sense + antisense	lpy-dpy larvae (>80%)[e]
hlh1D	intron 1	697	sense + antisense	wild type (<2% lpy-dpy)
myo-3 driven GFP transgenes[f]				makes nuclear GFP in body muscle
myo-3::NLS::gfp::lacZ				
gfpG	exons 2–5	730	sense	nuclear GFP-LacZ pattern of parent strain
			antisense	nuclear GFP-LacZ pattern of parent strain
			sense + antisense	nuclear GFP-LacZ absent in 98% of cells
lacZL	exon 12–14	830	sense + antisense	nuclear GFP-LacZ absent in >95% of cells
myo-3::MtLS::gfp				makes mitochondrial GFP in body muscle
gfpG	exons 2–5	730	sense	mitochondrial GFP pattern of parent strain
			antisense	mitochondrial GFP pattern of parent strain
			sense + antisense	mitochondrial GFP absent in 98% of cells
lacZL	exon 12–14	830	sense + antisense	mitochondrial GFP pattern of parent strain

Legend of Table 1

Each RNA was injected into 6–10 adult hermaphrodites ($0.5-1\times10^6$ molecules into each gonad arm). After 4–6 hours (to clear pre-fertilized eggs from the uterus) injected animals were transferred and eggs collected for 20–22 hours. Progeny phenotypes were scored upon hatching and subsequently at 12–24 hour intervals.

a: To obtain a semi-quantitative assessment of the relationship between RNA dose and phenotypic response, we injected each unc22A RNA preparation at a series of different concentrations. At the highest dose tested (3.6×10^6 molecules per gonad), the individual sense and antisense unc22A preparations produced some visible twitching (1% and 11% of progeny respectively). Comparable doses of ds-unc22A RNA produced visible twitching in all progeny, while a 120-fold lower dose of ds-unc22A RNA produced visible twitching in 30% of progeny.

b: unc22C also carries the intervening intron (43 nt).

c: fem1A also carries a portion (131 nt) of intron 10.

d: Animals in the first affected broods (laid at 4–24 hours after injection) showed movement defects indistinguishable from those of null mutants in unc-54. A variable fraction of these animals (25–75%) failed to lay eggs (another phenotype of unc-54 null mutants), while the remainder of the paralyzed animals were egg-laying positive. This may indicate partial inhibition of unc-54 activity in vulval muscles. Animals from later broods frequently exhibit a distinct partial loss-of-function phenotype, with contractility in a subset of body wall muscles.

e: Phenotypes of hlh-1 inhibitory RNA include arrested embryos and partially elongated L1 larvae (the hlh-1 null phenotype) seen in virtually all progeny from injection of ds-hlh1A and about half of the affected animals from ds-hlh1B and ds-hlh1C) and a set of less severe defects (seen with the remainder of the animals from ds-hlh1B and ds-hlh1C). The less severe phenotypes are characteristic of partial loss of function for hlh-1.

f: The host for these injections, PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ. This allows simultaneous assay for inhibition of gfp (loss of all fluorescence) and lacZ (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. ds-gfpG caused a loss of GFP in all but 0–3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0–5 additional bodywall muscles and in the eight vulval muscles.

TABLE 2

Effect of injection point on genetic inbibition in injected animals and their progeny

dsRNA	Site of injection	Injected animal phenotype	Progeny Phenotype
None	gonad or body cavity	no twitching	no twitching
None	gonad or body cavity	strong nuclear & mitochondrial GFP	strong nuclear & mitochondrial GFP
unc22B	Gonad	weak twitchers	strong twitchers
unc22B	Body Cavity Head	weak twitchers	strong twitchers
unc22B	Body Cavity Tail	weak twitchers	strong twitchers
gfpG	Gonad	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
gfpG	Body Cavity Tail	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
lacZL	Gonad	lower nuclear GFP	rare or absent nuclear GFP
lacZL	Body Cavity Tail	lower nuclear GFP	rare or absent nuclear GFP

TABLE 3

C. elegans can respond in a gene-specific manner to environmental dsRNA.

Bacterial Food	Movement	Germline Phenotype	GFP-Transgene Expression
BL21(DE3)	0% twitch	<1% female	<1% faint GFP
BL21(DE3) [fem-1 dsRNA]	0% twitch	43% female	<1% faint OFP
BL21(DE3) [unc22 dsRNA]	85% twitch	<1% female	<1% faint GFP
BL21(DE3) [gfp dsRNA]	0% twitch	<1% female	12% faint GFP

TABLE 4

Effects of bathing *C. elegans* in a solution containing dsRNA.

dsRNA	Biological Effect
unc-22	Twitching (similar to partial loss of unc-22 function)
pos-1	Embryonic arrest (similar to loss of pos-1 function)
sqt-3	Shortened body (Dpy) (similar to partial loss of sqt-3 function)

In Table 2, gonad injections were carried out into the GFP reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ. This allowed simultaneous assay of inhibition with gfp (fainter overall fluorescence), lacZ (loss of nuclear fluorescence), and unc-22 (twitching). Body cavity injections were carried out into the tail region, to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior region of the body cavity. An equivalent set of injections was also performed into a single gonad arm. For all sites of injection, the entire progeny brood showed phenotypes identical to those described in Table 1. This included progeny produced from both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could in part be due to the persistence of products already present in the injected adult. After ds-unc22B injection, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21/21) of these animals. Similar effects were seen with ds-unc22A. Injections of ds-gfpG or ds-lacZL produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of ds-gfpG and ds-lacZL produced no twitching, while injections of ds-unc22A produced no change in GFP fluorescence pattern.

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the present invention.

We claim:

1. A method to inhibit expression of a target gene in a cell in vitro comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA is a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence which corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence which is complementary to the nucleotide sequence of the target gene, wherein the first and the second ribonucleotide strands are separate complementary strands that hybridize to each other to form said double-stranded molecule, and the double-stranded molecule inhibits expression of the target gene.

2. The method of claim **1** in which the target gene is a cellular gene.

3. The method of claim **1** in which the target gene is an endogenous gene.

4. The method of claim **1** in which the target gene is a transgene.

5. The method of claim **1** in which the target gene is a viral gene.

6. The method of claim **1** in which the cell is from an animal.

7. The method of claim **1** in which the cell is from a plant.

8. The method of claim **6** in which the cell is from an invertebrate animal.

9. The method of claim **8** in which the cell is from a nematode.

10. The method of claim **1** in which the first ribonucleotide sequence comprises at least 25 bases which correspond to the target gene and the second ribonucleotide sequence comprises at least 25 bases which are complementary to the nucleotide sequence of the target gene.

11. The method of claim **1** in which the target gene expression is inhibited by at least 10%.

12. A method to inhibit expression of a target gene in an invertebrate organism comprising:

(a) providing an invertebrate organism containing a target cell, wherein the target cell contains the target gene and the target cell is susceptible to RNA interference, and the target gene is expressed in the target cell;

(b) contacting said invertebrate organism with a ribonucleic acid (RNA), wherein the RNA is a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence which corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence which is complementary to the nucleotide sequence of the target gene, wherein the first and the second ribonucleotide sequences are separate complementary strands that hybridize to each other to form the double-stranded molecule; and

(c) introducing the RNA into the target cell, thereby inhibiting expression of the target gene.

13. The method of claim **12** in which the organism is a nematode.

14. The method of claim **13** in which a formulation comprised of the RNA is applied on or adjacent to a plant, and disease associated with nematode infection of the plant is thereby reduced.

15. The method of claim **12** in which said double-stranded ribonucleic acid structure is at least 25 bases in length and each of the ribonucleic acid strands is able to specifically hybridize to a deoxyribonucleic acid strand of the target gene over the at least 25 bases.

16. The method of claim **12** in which the expression of the target gene is inhibited by at least 10%.

17. The method of claim **12** in which the RNA is introduced within a body cavity of the organism and outside the target cell.

18. The method of claim **12** in which the RNA is introduced by extracellular injection into the organism.

19. The method of claim **12** in which the organism is contacted with the RNA by feeding the organism food containing the RNA.

20. The method of claim **19** in which the food comprises a genetically-engineered host transcribing the RNA.

21. The method of claim **12** in which at least one strand of the RNA is produced by transcription of an expression construct.

22. The method of claim **21** in which the organism is a nematode and the expression construct is contained in a plant, and disease associated with nematode infection of the plant is thereby reduced.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,506,559 B1
DATED : January 14, 2003
INVENTOR(S) : Andrew Fire et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [73] Assignee, should read:
-- [73] Assignee: **The Carnegie Institution of Washington**, Washington, DC (US); **The University of Massachusetts**, Boston, Massachusetts (US) --

Signed and Sealed this

Sixteenth Day of September, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

US006506559B1

(12) United States Patent

Fire et al.

(10) Patent No.: US 6,506,559 B1
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(54) GENETIC INHIBITION BY DOUBLE-STRANDED RNA

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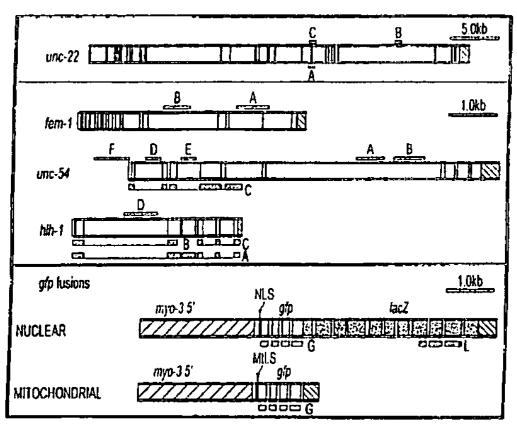
(57) **ABSTRACT**

A process is provided of introducing an RNA into a living cell to inhibit gene expression of a target gene in that cell. The process may be practiced ex vivo or in vivo. The RNA has a region with double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequences of the duplex region of the RNA and of a portion of the target gene are identical. The present invention is distinguished from prior art interference in gene expression by antisense or triple-strand methods.

22 Claims, 5 Drawing Sheets



The RNA may comprise one or more strands of polymerized ribonucleotide;

The double-stranded structure may be formed by a single self-complementary RNA strand

Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus).

be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript

The length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases.

The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus.

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects.

The double-stranded structure may be formed by a single self-complementary RNA strand

. RNA duplex formation may be initiated either inside or outside the cell.

RNA may be synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene in vivo or an expression construct, a regulatory

the duplex region of the RNA may

region (e.g., promoter, enhancer, silencer, splice donor and

acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands).

the duplex RNA can be produced by in vivo or in vitro transcription from an expression construct

strategy, single copies of the T7 promoter were used to drive expression of an inverted-duplication for a segment of the target gene, either unc-22 or gfp. This was comparably effective.

As an alternative

1. A method to inhibit expression of a target gene in a cell in vitro comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA is a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence which corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence which is complementary to the nucleotide sequence of the target gene, wherein the first and the second ribonucleotide strands are separate complementary strands that hybridize to each other to form said double-stranded molecule, and the double-stranded molecule inhibits expression of the target gene.

10. The method of claim 1 in which the first ribonucleotide sequence comprises at least 25 bases which correspond to the target gene and the second ribonucleotide sequence comprises at least 25 bases which are complementary to the nucleotide sequence of the target gene.

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(57) Abstract

The invention provides improved antisense oligonucleotides that are resistant to nucleolytic degradation. Such oligonuclotides are called self-stabilized oligonucleotides and comprise two regions: a target hybridizing region having a nucleotide sequence complementary to a nucleic acid sequence that is from a virus, a pathogenic organism, or a cellular gene; and a self-complementary region having an oligonucleotide sequence complementary to a nucleic acid sequence that is within the self-stabilized oligonucleotide.

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SELF-STABILIZED OLIGONUCLEOTIDES AS THERAPEUTIC AGENTS.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to novel therapeutic agents used in the antisense oligonucleotide therapeutic approach. More particularly, the invention relates to improved antisense oligonucleotides that have increased resistance to nucleases.

2. Summary of the Related Art

The antisense oligonucleotide based therapeutic principle provides an attractive strategy for rationally designing antiviral drugs and chemotherapeutic agents against other pathogens, and against disease conditions resulting from disorders of gene expression. The therapeutic principle relies upon specific binding between a target nucleic acid sequence and a complementary oligonucleotide. Several publications have demonstrated the efficacy of complimentary oligonucleotides in inhibiting gene expression by such specific binding.

Zamecnik and Stephenson, Proc. Natl. Acad. Sci. USA 75: 285-288 (1978) discloses specific inhibition of Rous Sarcoma Virus replication in infected chicken fibroblasts by a 13-mer synthetic oligodeoxynucleotide that is complementary to part of the viral genome.

Zamecnik et al., Proc. Natl. Acad. Sci. USA 83: 4143-4146 (1986) discloses inhibition of replication and expression of human immunodeficiency virus (HIV-1, then called HTLV-III) in cultured cells by synthetic oligonucleotide phosphodiesters complementary to viral RNA.

More recently, it has been reported that oligonucleotides having greater resistance to nucleolytic degradation than oligonucleotide phosphodiesters are more effective as antisense oligonucleotides. Agrawal, Tibtech 10: 152-158 (1992) has extensively reviewed the use of modified oligonucleotides as antiviral agents.

Sarin et al., Proc. Natl. Acad. Sci. USA 85: 7448-7451 (1988) teaches that oligodeoxynucleoside methylphosphonates are more active as inhibitors of HIV-1 than conventional oligodeoxynucleotides.

Agrawal et al., Proc. Natl. Acad. Sci. USA 85: 7079-7083 (1988) teaches that oligonucleotide phosphorothioates and various oligonucleotide phosphoramidates are more effective at inhibiting HIV-1 than conventional oligodeoxynucleotides.

Agrawal et al., Proc. Natl. Acad. Sci. USA 86: 7790-7794 (1989) discloses the advantage of oligonucleotide phosphorothioates in inhibiting HIV-1 in early and chronically infected cells.

An additional characteristic that renders oligonucleotides more effective as antisense agents is the ability to activate RNase H. Thus, oligonucleotide phosphorothioates, which are both resistant to nucleolytic degradation and activators of RNase H, are effective as inhibitors of HIV-1 and several other viruses.

Gao et al. Antimicrob. Agents and Chem. 34: 808 (1990) discloses inhibition of HSV by oligonucleotide phosphorothioates.

Storey et al., Nucleic Acids Res. 19: 4109 (1991) discloses inhibition of HPV by oligonucleotide phosphorothioates.

Leiter et al., Proc. Natl. Acad. Sci. USA 87: 3430 (1990) discloses inhibition of influenza virus by oligonucleotide phosphorothioates.

Unfortunately, oligonucleotide phosphorothioates increase resistance to nucleolytic degradation but do not provide complete resistance in vivo.

Agrawal et al., Proc. Natl. Acad. Sci. USA 88: 7595-7599 (1991) teaches that oligonucleotide phosphorothioates are extensively degraded from the 3' end in mice.

In addition, oligonucleotide phosphorothioates form less stable duplexes between the oligonucleotide and target than oligodeoxynucleotides phosphodiesters. To overcome these deficiencies, oligonucleotides having cap structures at the 3' terminus have been developed. Agrawal and Goodchild, Tetrahedron Lett. 28: 3539-3542 (1987) discloses the use of oligodeoxynucleoside methylphosphonates as 5' and 3' capping agents. Shaw et al., Nucleic Acids Res. 19: 747-750 (1991) discloses oligodeoxynucleotide phosphodiesters having blocking structures at the 3' end.

Temsamani et al., in Antisense Strategies, Annals of New York Academy of Sciences (in press) (1992) discloses 3' capped oligonucleotide phosphorothioates.

Even these nuclease resistant 3' capped oligonucleotides can become degraded eventually as the 3' capped end of these oligonucleotides is slowly digested by a combination of endonuclease and exonuclease activities.

There is, therefore, a need for oligonucleotides that form stable duplexes, resist nucleolytic degradation and activate RNase H, without the disadvantages of oligonucleotides that are known in the art. Ideally, such oligonucleotides should resist even the combined effect of endonucleases and exonucleases, should stably pair with target sequences at physiological temperatures,

should activate Rnase H and should produce only nucleosides as degradation products.

Oligonucleotides having self-complementary structures that can result in hairpin formation are known in the art.

Germann et al., Biochemistry 24: 5698-5702 (1985) discloses a partially self-complementary 24-mer oligonucleotide, $d(GC)_5 T_4(CG)_5$, that undergoes a ß-DNA to Z-DNA transition.

Hilbers et al., Biochimie 67: 685-695 (1985) discusses the dynamics of hairpin formation in a partially self-complementary oligonucleotide, $dATCCTAT_nTAGGAT$.

Neither of these physical studies related to either oligonucleotide stability or to therapeutic use of oligonucleotides.

Thus, the prior art is devoid of any teaching or suggestion about using self-complementary oligonucleotides in the antisense oligonucleotide therapeutic approach, nor does it discuss the use of hairpin formation as a means of rendering an oligonucleotide resistant to nucleolytic degradation.

BRIEF SUMMARY OF THE INVENTION

The invention relates to novel therapeutic agents used in the antisense oligonucleotide therapeutic approach. The invention provides improved antisense oligonucleotides that are resistant to nucleolytic degradation. Oligonucleotides according to the invention resist nucleolytic degradation, including the combined effect of endonucleases and exonucleases. Oligonucleotides according to the invention form stable hybrids with target sequences under physiological conditions, activate RNase H and produce only nucleosides as degradation products.

The advantages of oligonucleotides according to the invention, known as self-stabilized oligonucleotides, arise from the presence of two structural features: a target hybridizing region and a self-complementary region. The target hybridizing region comprises an oligonucleotide sequence that is complementary to a nucleic acid sequence that is from a plant or animal virus, a pathogenic organism, or a cellular gene or gene transcript, the abnormal gene expression or product of which results in a disease state. The self-complementary region comprises an oligonucleotide sequence that is complementary to a nucleic acid sequence within the oligonucleotide. Thus, at least when the oligonucleotide is not hybridized to a target nucleic acid sequence, the oligonucleotide forms a totally or partially double-stranded structure that is resistant to nucleolytic degradation. Since the inherent structure of these molecules confers resistance to nucleases, it is not necessary to use modified internucleotide linkages to confer such resistance, although of course, modified linkages may be used. Thus, the use of oligonucleotide phosphodiesters or oligonucleotide phosphorothioates, both of which are degraded <u>in vivo</u>, is made feasible by oligonucleotides according to the invention. This

results in oligonucleotides that activate RNase H, an important feature for the antisense therapeutic compound. Also, the use of oligonucleotide phosphodiesters provides more stable hybridization between therapeutic oligonucleotides and target sequences. Finally, degradation of such oligonucleotides results only in nucleotide breakdown products, thus minimizing potential toxicity. These advantages result in a superior therapeutic oligonucleotide.

The invention further provides self-stabilized ribozymes, since the self-complementary motif of the invention can be conveniently used with ribonucleotides. Such ribozymes according to the invention have generally typical ribozyme structure, except that they have a self-complementary region at or near the 5' or 3' end. This region confers nuclease resistance upon the ribozymes, making them more stable than ribozymes that are known in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a self-stabilized oligonucleotide according to the invention in hairpin and hybridized configurations.

Figure 2 illustrates a self-stabilized oligonucleotide according to the invention in hammer-like configuration.

Figure 3 shows results of duplex stability studies for hybridization between oligonucleotides or self-stabilized oligonucleotides and complementary target oligonucleotides.

Figure 4 shows results of 3'-exonuclease treatment of oligonucleotides.

Figure 5 shows the structure of self-stabilized oligonucleotides used in Examples 1-4.

Figure 6 shows a mechanism of therapeutic action of self-stabilized oligonucleotides.

Figure 7 shows a self-stabilized ribozyme according to the invention. This example of a self-stabilized ribozyme according to the invention is complementary to the HIV gag region and results in the cleavage of a HIV gag mRNA.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to novel therapeutic agents that are useful in the treatment of virus infections, infections by pathogenic organisms, and diseases arising from abnormal gene expression or gene products.

In a first aspect, the invention provides therapeutic self-stabilized oligonucleotides that are more resistant to nucleolytic degradation than oligonucleotides that are known in the art. For purposes of the invention, the term oligonucleotide includes polymers of ribonucleotides, deoxyribonucleotides, or both, with ribonucleotide and/or deoxyribonucleotide monomers being connected together via 5' to 3' linkages which may include any of the linkages that are known in the antisense oligonucleotide art. In addition, the term oligonucleotides includes such molecules having modified nucleic acid bases and/or sugars, as well as such molecules having added substituents, such as diamines, cholesteryl or other lipophilic groups. Certain preferred combinations of monomers and inter-monomer linkages are discussed in greater detail below.

Oligonucleotides according to the invention are generally characterized by having two regions: a target hybridizing region and a self-complementary region. A first embodiment of a self-stabilized oligonucleotide according to the invention is shown in Figure 1. In this embodiment, the target hybridizing region is shown as connected rectangular squares, and the self-complementary region is shown as connected circles. The complementary nucleic acid sequence in a target RNA molecule is represented by connected diamonds. Hydrogen bonding between nucleotides is indicated by dots. The oligonucleotide is stabilized, i.e., rendered resistant to nucleolytic degradation from the 5' or 3' end by base-pairing between the target hybridizing region and the

self-complementary regions and/or by base-pairing between complementary sequences within the self-complementary region. When the oligonucleotide encounters a nucleic acid molecule having a complementary nucleic acid sequence, base-pairing between the target hybridizing region and the self-complementary region of the oligonucleotide is disrupted and replaced by base-pairing between the target hybridizing region of the oligonucleotide and the complementary nucleic acid sequence of the target nucleic acid molecule. This disruption and replacement of base-pairing takes place because the intermolecular base-paired structure formed by the hybrid between the target nucleic acid sequence and the target hybridizing region is more thermodynamically stable than the intra-molecular base-paired structure formed by the self-complementary oligonucleotide. This phenomenon is illustrated in Figure 3 and discussed in greater detail in Example 4.

A second embodiment of an oligonucleotide according to the invention operates in a similar way as the first embodiment, but forms a different structure upon self-complementary base-pairing. This alternative embodiment forms a hammer-like structure as shown in Figure 2. In this embodiment, the self-complementary region contains oligonucleotide sequences that can base pair with other oligonucleotide sequences within the self-complementary region. The self complementary region may also contain oligonucleotide sequences that are complementary to the target hybridizing region.

The target hybridizing region of an oligonucleotide according to the invention has an oligonucleotide sequence that is complementary to a nucleic acid sequence that is from a virus, a pathogenic organism, or a cellular gene or gene transcript, the abnormal gene expression or product of which results in a disease state. Preferably the target hybridizing region is from

about 8 to about 50 nucleotides in length. For purposes of the invention, the term "oligonucleotide sequence that is complementary to a nucleic acid sequence" is intended to mean an oligonucleotide sequence (2 to about 50 nucleotides) that hybridizes to the nucleic acid sequence under physiological conditions, e.g., by Watson-Crick base paring (interaction between oligonucleotide and single-stranded nucleic acid) or by Hoogsteen base pairing. (interaction between oligonucleotide and double-stranded nucleic acid) or by any other means. Such hybridization under physiological conditions is measured as a practical matter by observing interference with the function of the nucleic acid sequence.

The nucleic acid sequence to which the target hybridizing region of an oligonucleotide according to the invention is complementary will vary, depending upon the disease condition to be treated. In many cases the nucleic acid sequence will be a virus nucleic acid sequence. The use of antisense oligonucleotides to inhibit various viruses is well known, and has recently been reviewed in Agrawal, Tibtech 10:152-158 (1992). Viral nucleic acid sequences that are complementary to effective antisense oligonucleotides have been described for many viruses, including human immunodeficiency virus type 1 (U.S. Patent No. 4,806,463, the teachings of which are herein incorporated by reference), Herpes simplex virus (U.S. Patent No. 4,689,320, the teachings of which are hereby incorporated by reference), Influenza virus (U.S. Patent No. 5,XXX,XXX; Ser. No. 07/516,275, allowed June 30, 1992; the teachings of which are hereby incorporated by reference), and Human papilloma virus (Storey et al., Nucleic Acids Res. 19:4109-4114 (1991)). Sequences complementary to any of these nucleic acid sequences can be used for the target hybridizing region of oligonucleotides according to the invention, as can be oligonucleotide sequences

complementary to nucleic acid sequences from any other virus. Additional viruses that have known nucleic acid sequences against which antisense oligonucleotides can be prepared include Foot and Mouth Disease Virus (See Robertson et al., J. Virology 54: 651 (1985); Harris et al., J. Virology 36: 659 (1980)), Yellow Fever Virus (See Rice et al., Science 229: 726 (1985)), Varicella-Zoster Virus (See Davison and Scott, J. Gen. Virology 67: 2279 (1986), and Cucumber Mosaic Virus (See Richards et al., Virology 89: 395 (1978)).

Alternatively, the target hybridizing region of oligonucleotides according to the invention can have an oligonucleotide sequence complementary to a nucleic acid sequence of a pathogenic organism. The nucleic acid sequences of many pathogenic organisms have been described, including the malaria organism, Plasmodium falciparum, and many pathogenic bacteria. Oligonucleotide sequences complementary to nucleic acid sequences from any such pathogenic organism can form the target hybridizing region of oligonucleotides according to the invention. Examples of pathogenic eukaryotes having known nucleic acid sequences against which antisense oligonucleotides can be prepared include Trypanosoma brucei gambiense and Leishmania (See Campbell et al., Nature 311: 350 (1984)), Fasciola hepatica (See Zurita et al., Proc. Natl. Acad. Sci. USA 84: 2340 (1987). Antifungal oligonucleotides can be prepared using a target hybridizing region having an oligonucleotide sequence that is complementary to a nucleic acid sequence from, e.g., the chitin synthetase gene, and antibacterial oligonucleotides can be prepared using, e.g., the alanine racemase gene.

In yet another embodiment, the target hybridizing region of oligonucleotides according to the invention can have an oligonucleotide sequence complementary to a

cellular gene or gene transcript, the abnormal expression or product of which results in a disease state. The nucleic acid sequences of several such cellular genes have been described, including prion protein (Stahl and Prusiner, FASEB J. 5: 2799-2807 (1991)), the amyloid-like protein associated with Alzheimer's disease (U.S. Patent No. 5,015,570, the teachings of which are hereby incorporated by reference), and various well-known oncogenes and proto-oncogenes, such as c-myb, c-myc, c-abl, and n-ras. In addition, oligonucleotides that inhibit the synthesis of structural proteins or enzymes involved largely or exclusively in spermatogenesis, sperm motility, the binding of the sperm to the egg or any other step affecting sperm viability may be used as contraceptives for men. Similarly, contraceptives for women may be oligonucleotides that inhibit proteins or enzymes involved in ovulation, fertilization, implantation or in the biosynthesis of hormones involved in those processes.

Hypertension can be controlled by oligodeoxynucleotides that suppress the synthesis of angiotensin converting enzyme or related enzymes in the renin/angiotensin system; platelet aggregation can be controlled by suppression of the synthesis of enzymes necessary for the synthesis of thromboxane A2 for use in myocardial and cerebral circulatory disorders, infarcts, arteriosclerosis, embolism and thrombosis; deposition of cholesterol in arterial wall can be inhibited by suppression of the synthesis of fattyacryl co-enzyme A: cholesterol acyl transferase in arteriosclerosis; inhibition of the synthesis of cholinephosphotransferase may be useful in hypolipidemia.

There are numerous neural disorders in which hybridization arrest can be used to reduce or eliminate adverse effects of the disorder. For example, suppression of the synthesis of monoamine oxidase can be

used in Parkinson's disease; suppression of catechol o-methyl transferase can be used to treat depression; and suppression of indole N-methyl transferase can be used in treating schizophrenia.

Suppression of selected enzymes in the arachidonic acid cascade which leads to prostaglandins and leukotrienes may be useful in the control of platelet aggregation, allergy, inflammation, pain and asthma.

Suppression of the protein expressed by the multidrug resistance (mdr) gene, which is responsible for development of resistance to a variety of anti-cancer drugs and is a major impediment in chemotherapy may prove to be beneficial in the treatment of cancer. Oligonucleotide sequences complementary to nucleic acid sequences from any of these genes can be used for the target hybridizing region of oligonucleotides according to the invention, as can be oligonucleotide sequences complementary to any other cellular gene or gene transcript, the abnormal expression or product of which results in a disease state.

Antisense regulation of gene expression in plant cells has been described in U.S. Patent No. 5,107,065, the teachings of which are hereby incorporated by reference.

In a second aspect, the invention provides nuclease resistant oligonucleotides that activate RNase H. The target hybridizing region of oligonucleotides according to the invention may contain ribonucleotides, deoxyribonucleotides or any analogs of ribonucleotides or deoxyribonucleotides. In one preferred embodiment, this region is composed of ribonucleotides. In another preferred embodiment, this region is composed of deoxyribonucleotides. In yet another preferred embodiment, this region comprises a mixture of ribonucleotides and deoxyribonucleotides. An additional

preferred embodiment has a target hybridizing region comprising oligonucleotide phosphodiesters, phosphorothioates, or phosphorodithioates, or mixtures or these with ribonucleotides or deoxyribonucleotides. These preferred embodiments all provide for the activation of RNase H, as long as four or more contiguous deoxyribonucleotide phosphodiesters, phosphorothioates, or phosphorodithioates are present. Of course, other embodiments employing target hybridizing regions that do not activate RNase H can also be made.

Synthesis procedures for each of these embodiments are well known in the art. Both oligodeoxyribonucleotide phosphodiesters and oligodeoxyribonucleotide phosphorothioates and their analogs can be synthesized by the H-phosphonate approach described in U.S. Patent No. 5, , ,(Ser. No. 07/334,679; allowed on March 19, 1992), the teachings of which are hereby incorporated by reference. The H-phosphonate approach can also be used to synthesize oligoribonucleotides and oligoribonucleotide analogs, as described in Agrawal and Tang, Tetrahedron Lett. 31: 7541-7544 (1990). Synthesis of oligonucleotide phosphorodithioates is also known in the art.

Of course, many other embodiments are possible, and those skilled in the art will recognize that other analogs or combinations of analogs can be used in the target hybridizing region of oligonucleotides according to the invention. Such analogs are characterized by having internucleotide linkages other than the natural phosphodiester linkage. The synthesis of many such analogs is well known in the art, including analogs having alkylphosphonate, (Agrawal and Goodchild, Tetrahedron Lett. 28: 3539-3542 (1987)) or phosphoramidate (Agrawal et al., Proc. Natl. Acad. Sci. USA 85: 7079-7083 (1988)) linkages.

The second significant region of self-stabilized oligonucleotides according to the invention is the self-complementary region. The self-complementary region contains oligonucleotide sequences that are complementary to other oligonucleotide sequences within the oligonucleotide. These other oligonucleotide sequences may be within the target hybridizing region or within the self-complementary region, or they may span both regions. The complementary sequences form base pairs, resulting in the formation of a hairpin structure, as shown in Figure 1, or a hammer-like structure, as shown in Figure 2. Either the hairpin structure or the hammer-like structure can have loops resulting from non-base-paired nucleotides, as shown in Figure 1 for the hairpin structure, or can be devoid of such loops, as shown in Figure 2 for the hammer-like structure. The number of base-pairs to be formed by intra-molecular hybridization involving the self-complementary region may vary, but should be adequate to maintain a double-stranded structure so that the 3' end is not accessible to endonucleases. Generally, about 4 or more base-pairs will be necessary to maintain such a double-stranded structure. In a preferred embodiment, there are about 10 intramolecular base-pairs formed in the self-stabilized oligonucleotide, with the 10 base pairs being consecutive and involving the 3'-most nucleotides. Of course, the intra-molecular base-pairing can be so extensive as to involve every nucleotide of the oligonucleotide. Preferably, this will involve a self-complementary region of about 50 nucleotides or less.

In one preferred embodiment the self-complementary region may be connected to the target hybridizing region by a suitable non-nucleic acid linker. Examples of such linkers include substituted or unsubstituted alkyl groups. In one most preferred embodiment the linker is a (ethylene glycol)$_{1-6}$ linker. At the larger size for

this linker, the synthesis may be conveniently carried out by using commercially available triethylene glycol that has a dimethyltrityl protective group at one end and a cyanoethylphosphoramidite group at the other end.

The self-complementary region may contain ribonucleotides, deoxyribonucleotides, analogs of ribonucleotides or deoxyribonucleotides having artificial linkages, or combinations of any of the above. The ability to activate RNase H is not important for the self-complementary region, so nucleotides having artificial linkages that do not activate RNase H can be used in this region without diminishing the effectiveness of the oligonucleotide. Thus, in addition to phosphodiester and phosphorothioate or phosphorodithioate linkages, this region may also or alternatively contain phosphoramidate (including N-substituted phosphoramidates), alkylphosphonate, alkylphosphonothioate linkages as well as non-phosphate containing linkages, such as sulfone, sulfate, and keto linkages. Of course, in non-RNase H activating embodiments of self-stabilized oligonucleotides according to the invention, any of these linkages can be used in the target hybridizing region as well.

In one preferred embodiment, the self-stabilized oligonucleotide is rendered hyperstabilized. This may be accomplished by incorporating into the self-complementary region one or more ribonucleotides or 2'-O-Me-ribonucleotides, wherein the complementary portion of the target hybridizing region is DNA. Alternatively, the complementary region of the target hybridizing region may contain ribonucleotides or 2'-O-Me-ribonucleotides, and the self-complementary region may contain DNA. These oligonucleotides will be hyperstabilized because the interaction between DNA and RNA is more stable than the interaction between DNA and DNA. Yet another way in which the self-complementary region (and/or the linker

region) may be modified to yield a hyperstabilized self-stabilized oligonucleotide is to incorporate one or more intercalating agent molecule. These oligonucleotides are hyperstabilized because the intercalating agent stabilizes the hybrid formed between the self-complementary region and the target hybridizing region. Any intercalating agent is acceptable for this purpose. Preferred intercalating agents include acridine and ethidium. Oligonucleotides containing acridine are readily prepared by using the commercially available acridine-ON phosphoramidite, or 3'-acridine-ON CPG (Clontech Laboratories, Inc.).

In a third aspect, the invention provides ribozymes that are more stable than ribozymes that are known in the art. Ribozymes are catalytic RNA molecules that cleave internucleotide bonds. The stability of such ribozymes according to the invention is provided by the incorporation of a self-complementary region at or near the 5' or 3' end of the ribozyme molecule. This self-complementary region results in the formation of a hairpin or hammer-like structure, thus rendering the 5' or 3' end of the molecule double-stranded, which causes the ribozyme molecule to resist nucleolytic degradation. The structure and function of ribozymes is generally taught in U.S. Patent No. 4,987,071, the teachings of which are hereby incorporated by reference.

In a fourth aspect, the invention provides a method for inhibiting the gene expression of a virus, a pathogenic organism or a cellular gene, the method comprising the step of providing self-stabilized oligonucleotides or ribozymes according to the invention to cells infected with the virus or pathogenic organism in the former two cases or to cells generally in the latter case.

In a fifth aspect, the invention provides a method of treating a diseased human or animal in which the disease results from infection with a virus or pathogenic organism, or from the abnormal expression or produce of a cellular gene. The method comprises administering self-stabilized oligonucleotides according to the invention in a pharmaceutically acceptable carrier to the diseased human or animal. Preferably, the routes of such administration will include oral, intranasal, rectal and topical administration. In such methods of treatment according to the invention the self-stabilized oligonucleotides may be administered in conjunction with other therapeutic agents, e.g., AZT in the case of AIDS.

A variety of viral diseases may be treated by the method of treatment according to the invention, including AIDS, ARC, oral or genital herpes, papilloma warts, flu, foot and mouth disease, yellow fever, chicken pox, shingles, HTLV-leukemia, and hepatitis. Among fungal diseases treatable by the method of treatment according to the invention are candidiasis, histoplasmosis, cryptococcocis, blastomycosis, aspergillosis, sporotrichosis, chromomycosis, dematophytosis and coccidioidomycosis. The method can also be used to treat rickettsial diseases (e.g., typhus, Rocky Mountain spotted fever), as well as sexually transmitted diseases caused by Chlamydia trachomatis or Lymphogranuloma venereum. A variety of parasitic diseases can be treated by the method according to the invention, including amebiasis, Chegas' disease, toxoplasmosis, pneumocystosis, giardiasis, cryptosporidiosis, trichomoniasis, and Pneumocystis carini pneumonia; also worm (helminthic diseases) such as ascariasis, filariasis, trichinosis, schistosomiasis and nematode or cestode infections. Malaria can be treated by the method of treatment of the invention regardless of whether it is

caused by P. falciparum, P. vivax, P. orale, or P. malariae.

The infectious diseases identified above can all be treated by the method of treatment according to the invention because the infectious agents for these diseases are known and thus self-stabilized oligonucleotides according to the invention can be prepared, having a target hybridizing region that has an oligonucleotide sequence that is complementary to a nucleic acid sequence that is an essential nucleic acid sequence for the propagation of the infectious agent, such as an essential gene.

Other disease states or conditions that are treatable by the method according to the invention result from an abnormal expression or product of a cellular gene. These conditions can be treated by administration of self-stabilized oligonucleotides according to the invention, and have been discussed earlier in this disclosure.

The invention provides numerous advantages over oligonucleotides that are known in the art. First, the self-stabilized oligonucleotides according to the invention have a longer half-life than most known oligonucleotides, thereby lowering the dosage that will be required for therapeutic efficacy. Even greater resistance to nuclease degradation can be provided by using nuclease resistant internucleotide linkages near or cap structures at one or both ends of the oligonucleotide. Second, the enzymatic stability afforded by the base-paired structures involving the self-complementary sequences allows the use of oligonucleotide phosphodiesters, which otherwise are rapidly degraded. This provides the advantages of increased duplex stability and RNase H activation, which are not both provided by any nuclease resistant

oligonucleotide known in the art. The advantage of RNase H activation is retained when oligonucleotide phosphorothioates or phosphorodithioates are used. A third advantage is that the only degradation product of several embodiments of oligonucleotides according to the invention is nucleotides, e.g., nucleoside monophosphates and/or nucleoside monothiophosphates. Finally, the invention allows the use of either deoxyribonucleosides or ribonucleosides. The ability to use the latter makes the invention readily adaptable for use with ribozymes, for which enzymatic stability is critical.

The following examples are provided to further illustrate certain aspects of preferred embodiments of the invention, and are not intended to be limiting in nature.

EXAMPLE 1

Nuclease Resistance of Oligonucleotide Phosphodiesters

The oligonucleotides used in the study are shown in Figure 5. Oligonucleotide CMPD A is complementary to a portion of the gag region of HIV-1. Oligonucleotide CMPD B uses this same region as a target hybridizing region, but adds a 3' self-complementary region of 10 nucleotides. Oligonucleotides CMPD E and CMPD F are identical to CMPD B, except that the self-complementary regions of CMPD E and CMPD F are 6 and 4 nucleotides, respectively. Oligonucleotide CMPD G is identical to CMPD A, except that it has 10 mismatched nucleotides (T_{10}) added at its 3' end.

The oligonucleotides were tested for their relative resistance to 3' exonucleolytic degradation. For each oligonucleotide, 0.4 A_{260} units of oligonucleotide was lyophilized, dissolved in 0.5ml buffer (10mM Tris, 10mM $MgCl_2$, pH 8.5) and mixed with 5μl (1.5 milliunits) of

snake venom phosphodiesterase (SVPD). The mixture was incubated at 37°C in a thermally regulated cell and A_{260} was plotted against time. Oligonucleotide degradation was measured as function of increase in hyperchromicity.

The results of these experiments are shown in Table I, below. These results demonstrate that self-stabilized oligonucleotide phosphodiesters according to the invention are far more resistant to 3' exoncleolytic degradation than either oligonucleotide phosphodiesters or oligonucleotide phosphodiesters having a non-complementary tail.

In addition to the testing described above, the oligonucleotides were also subjected to DNA Polymerase I 3'-exonuclease digestion. As shown in Figure 4 the non-self-stabilized oligonucleotides, CMPDs A and G were digested to completion in 30 minutes, whereas self-stabilized CMPD B was only partly digested over 30 minutes.

TABLE I

HALF-LIFE OF OLIGONUCLEOTIDES

Oligonucleotide	$t_{1/2}$ for SVPD digestion
CMPD A	75 seconds
CMPD G	75 seconds
CMPD B	950 seconds

EXAMPLE 2

Nuclease Resistance of Oligonucleotide Phosphorothioates

To test the relative nuclease resistance of self-stabilized and non-self-stabilized oligonucleotide phosphorothioates, a DNA Polymerase I 3'- exonuclease activity assay was used, because of the slow degradation of oligonucleotide phosphorothioates by SVPD.

All oligonucleotides were labelled at the 5'- end with gamma-^{32}p-ATP and kinase. To a solution of 40 pmole

5'-labelled oligonucleotide in 20μl buffer (40 mM Tris. HCl pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 50 mM KCl, 50μg/ml BSA), 5 units DNA polymerase I was added and incubated at 37°C. Aliquots of 4μl were taken at 0, 30, 60, 120 minutes and were mixed with 6 μl stop solution (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue). The samples were analyzed by 15% acrylamide gel (urea) and autoradiography.

The results are shown in Figure 4. Phosphorothioate analog of CMPD A was digested to almost 50% within 4 hours. The phosphorothioate analog of CMPD B, however, was undegraded after 4 hours. Phosphorothioate analogs of CMPD E and F, which have 6 and 4 base pairs of self-complementary sequence, respectively were also found to be stable. Phosphorothioate analog of CMPD G, having extended structure, but no self-complementary region, was digested at same rate as CMPD A. These results demonstrate that self-stabilized oligonucleotide phosphorothioates are far more resistant to nucleolytic degradation than are non-self-stabilized oligonucleotide phosphorothioates.

EXAMPLE 3

Anti-HIV Activity Of Oligonucleotides

Self-stabilized and non-self stabilized oligonucleotide phosphodiesters were tested for their ability to inhibit HIV-1 in tissue culture. The oligonucleotides used in this study are shown in Figure 5.

H9 lymphocytes were infected with HIV-1 virions (≡0.01 - 0.1 $TCID_{50}$/cell) for one hour at 37°C. After one hour, unadsorbed virions were washed and the infected cells were divided among wells of 24 well plates. To the infected cells, an appropriate concentration (from stock solution) of oligonucleotide was added to obtain the required concentration in 2 ml medium. In a positive

control experiment ddC or AZT was added. The cells were then cultured for three days. At the end of three days, supernatant from the infected culture was collected and measured for p24 expression by ELISA. The level of expression of p24 was compared between oligonucleotide treated and untreated (no drug) infected cells.

Cytotoxicity of oligonucleotides was studied by culturing the cells with increasing concentration of oligonucleotide and by the trypan blue dye exclusion method.

The results of two experiments are shown in Table III, below.

TABLE III

Anti-HIV Activity of Oligonucleotides

Experiment 1

	Concentration (g/ml)	Inhibition of p24 (%)	% Cell Survival	IC_{50} (g/ml)
CMPD A	25	90	93	2
	5	89	103	
	1	15	94	
	0.2	26	97	
CMPD B	25	90	95	0.25
	5	85	92	
	1	84	94	
	0.2	46	103	
CMPD G	25	86	106	0.5
	5	86	105	
	1	81	106	
	0.2	0	109	
AZT	0.2	90	95	0.037μM
	0.04	73	98	
	0.08	44	104	
	.0016	6	108	

Experiment 2

	Concentration (g/ml)	Inhibition of p24 (%)	% Cell Survival	IC_{50} (g/ml)
CMPD A	5	66	93	2.8
	1	20	101	
	0.2	21	107	
	0.04	0	102	
CMPD B	5	93	89	0.35
	1	81	99	
	0.2	33	103	
	0.04	0	104	
CMPD E	5	89	93	0.45
	1	41	100	
	0.2	19	99	
	0.04	0	102	
CMPD F	5	89	93	1.5
	1	41	100	
	0.2	19	99	
	0.04	0	102	
AZT	0.2	89	93	0.1μm
	0.04	65	98	
	0.008	5	101	
	0.0016	6	103	

All self-stabilized oligonucleotides exhibited greater anti-HIV activity than CMPD A, the non-self-stabilized oligonucleotide. Greatest activity was observed for the self-stabilized oligonucleotide having 10 self-complementary nucleotides, which exhibited nearly ten times the activity of the oligonucleotide phosphodiester. The oligonucleotide CMPD G, which has a poly T tail, also showed some increase in activity,

probably as a result of stabilization from hybridization with polyA from mRNA in the H9 cells.

The probable mechanism of action of the CMPD B oligonucleotide is shown in Figure 6. The oligonucleotide enters the cell in a partially double-stranded form as a result of intramolecular base-paring involving the self-complementary region. As the oligonucleotide encounters on HIV RNA molecule having a nucleic acid sequence that is complementary to the oligonucleotide sequence of the target hybridizing region of the oligonucleotide, hybridization occurs between the HIV RNA and the target hybridizing region. This hybridization disrupts the intramolecular hybridization involving the self-complementary region. RNase H activity then cleaves the HIV RNA, allowing the oligonucleotide to once again self-stabilize by intramolecular base-pairing.

To test the relative anti-HIV activity of additional oligonucleotide structures, the above experiment was repeated using additional oligonucleotides, as well as the oligonucleotides described in Experiments 1 & 2. The additional oligonucleotides are shown in Figure 5. These additional oligonucleotides were CMPD C, in which the self-complementary region is complementary to the oligonucleotide through its 5' end; CMPD D, which has a 8 nucleotide self-complementary region; and CMPD H, a 35 mer oligonucleotide having perfect complementarity to the HIV gag RNA, but no self-complementary region. The results of this third experiment are shown in Table IV, below.

These results demonstrate that fully self-complementary self-stabilized oligonucleotides are roughly equivalent in anti-HIV activity to partially self-complementary self-stabilized oligonucleotides. The results also show that four self-complementary nucleotides are adequate to confer enhanced efficacy.

TABLE IV
Anti-HIV Activity of Oligonucleotides

Experiment 3

	Concentration (g/ml)	Inhibition of p24 (%)	% Cell Survival	IC_{50} (g/ml)
CMPD A	5.0	92	97	1.7
	1.0	36	103	
	0.2	23	102	
	0.04	0	109	
CMPD B	5.0	95 (97)*	98 (97)*	0.5 (0.2)*
	1.0	61 (74)*	101 (102)*	
	0.2	33 (49)*	104 (103)*	
	0.04	0 (19)*	11 (106)*	
CMPD G	5.0	94	97	0.6
	1.0	68	104	
	0.2	11	109	
	0.04	12	110	
CMPD E	5.0	92	98	0.8
	1.0	55	101	
	0.2	13	103	
	0.04	0	107	
CMPD F	5.0	95	99	0.25
	1.0	64	102	
	0.2	48	104	
	0.04	22	109	
CMPD C	5.0	94	96	0.3
	1.0	76	101	
	0.2	39	103	
	0.04	17	106	

*Results of second independent screening.

	Concentration (μg/ml)	Inhibition of p24 (%)	% Cell Survival	IC_{50} (μg/ml)
CMPD H	5	92	93	0.26
	1	88	101	
	.2	43	98	
	.04	0	102	
CMPD D	5	80	93	0.4
	1	76	100	
	.2	30	108	
	.04	3	109	

EXAMPLE 4

Stability of Duplexes Between Self-Stabilized Oligonucleotides and Complementary Oligos

To test the stability of duplexes formed between self-stabilized oligonucleotides and complementary nucleic acid sequences hybridization studies were carried out. In a first study oligonucleotide CMPD A, which lacks self-complementary sequences, was mixed at room temperature with a complementary 25-mer oligonucleotide. The mixture was then gradually heated and increase in hyperchromicity was plotted against increase in temperature. In this study, the results of which are shown as a dotted line in Figure 3, the melting temperature of the duplex was found to be about 65°C.

In a second study CMPD B, having the same target hybridizing region as CMPD A and a 10 nucleotide self-complementary region, was mixed with the same 25-mer oligonucleotides at room temperature. The mixture was then gradually heated and increase in hyperchromicity was plotted against increase in temperature. The results are

shown as a solid line in Figure 3. This time, in addition to the melting observed at about 65°C, an earlier increase in hyperchromicity was observed at about 58°C, corresponding to the melting of the intra molecular hydrogen bonds of the hairpin structure. This result indicates that the intramolecular base pairing involving the self-complementary region is less thermodynamically stable than the intermolecular base pairing between the target hybridizing region and a complementary oligonucleotide.

To further test the increased stability of the intermolecular base pairing relative to the intramolecular base pairing, CMPD B was then mixed with the same complementary 25-mer oligonucleotide and heated to 80°C, then allowed to cool to room temperature. This mixture was then gradually heated and increase in hyperchromicity was plotted against increase in temperature. The results are shown as a dashed line in Figure 3. Only a single melting temperature of about 65°C was observed, indicating that the intermolecular base pairing between CMPD B and the complementary 25-mer oligonucleotide is favored in competition with intramolecular base pairing involving the self-complementary region.

These results demonstrate that self-stabilized oligonucleotides will hybridize to complementary nucleic acid sequences notwithstanding the presence of oligonucleotide sequences within the oligonucleotide that are complementary to the target hybridizing region. Since it is well known that certain types of oligonucleotide structures hybridize more stably than certain other types of oligonucleotide structures (e.g., RNA:DNA hybrids > DNA:DNA hybrids and phosphodiester - containing oligos > phosphorothioate methylphosphonate or phosphoramidate - containing oligos), these results also indicate that the preferential target hybridizing effect

may be enhanced by designing the self-stabilized oligonucleotide such that the hybridization between the target hybridizing region and the target sequence involves more stably pairing oligonucleotide structures than the hybridization involving the self-complementary region.

Those skilled in the art will recognize that self-complementary regions can be prepared according to the above teachings and combined with a wide variety of target hybridizing regions.

EXAMPLE 5

Hyperstabilized Self-Stabilized Oligonucleotides

To provide oligonucleotides having a more stable interaction between the self-complementary region and the target hybridizing region, oligodeoxynucleoside phosphodiesters or oligodeoxynucleoside phosphorothioates were prepared that had 2-O-Me-ribonucleosides in the self-complementary region. As shown in Table V below, such oligonucleotides had a hyperstabilized interaction between the self-complementary region and the target hybridizing region. Nevertheless, these oligonucleotides continued to favor formation of intermolecular hybrids with complementary DNA, relative to molecules containing intramolecular hybrids.

TABLE V

DUPLEX STABILITY OF SELF-STABILIZED OLIGONUCLEOTIDES HAVING 2-O-Me-RIBONUCLEOTIDES IN THE SELF-COMPLEMENTARY REGION

	TM	Complementary with DNA (25 mer)
5'-CTCTCGCACCCATCTCTCTCCTTCTGGAGA-3'	59°C	64.8°C
5'-CTCTCGCACCCATCTCTCTCCTTCTGGAGAG-3'	66°C	64.5°C
5'-CTCTCGCACCCATCTCTCTCCTTCTGGAGAGAG-3'	71°C	65°C

Another class of hyperstabilized self-stabilized oligonucleotides was prepared by covalently linking an acridine molecule to the terminus of the self-complementary region. These molecules also demonstrated hyperstability of the interaction between the target hybridizing region and the self-complementary region. Nevertheless, these molecules still preferentially formed intermolecular hybrids with complementary DNA, relative to forming intramolecular hybrids.

TABLE VI

DUPLEX STABILITY OF SELF- STABILIZED OLIGONUCLEOTIDES HAVING INTERCALATING AGENTS IN THE SELF-COMPLEMENTARY REGION

	TM	Complementary with DNA (25 mer)
5'-CTCTCGCACCCATCTCTCTCCTTCTX	N/A	67.5°C
5'-CTCTCGCACCCATCTCTCTCCTTCTGGX-3'	N/A	66.7°C
5'-CTCTCGCACCCATCTCTCTCCTTCTGGAGX-3'	65°C	66.3°C
5'-CTCTCGGACCCATCTCTCTCCTTCTGGAGAGX-3'	66.8°c	66.7°C

These results indicate that it is possible to construct hyperstabilized self-stabilized oligonucleotides having very stable interactions between the self-complementary region and the target hybridizing region, without interfering with the ability of the oligonucleotide to form intermolecular hybrids with a target nucleic acid.

We claim:

1. A therapeutic self-stabilized oligonucleotide comprising a target hybridizing region and a self-complementary region,

wherein the target hybridizing region comprises a oligonucleotide sequence complementary to a nucleic acid sequence that is from a virus, a pathogenic organism or a cellular gene, and wherein the self-complementary region comprises an oligonucleotide sequence complementary to a nucleic acid sequence that is within the therapeutic self-stabilized oligonucleotide.

2. A therapeutic self-stabilized oligonucleotide according to claim 1, wherein the target hybridizing region comprises four or more contiguous deoxyribonucleotide phosphodiesters, phosphorothioates, or phosphorodithioates.

3. A therapeutic self-stabilized oligonucleotide according to claim 1, wherein the self-complementary region comprises nucleotides selected from the group consisting of: deoxyribonucleotide or ribonucleotide phosphodiesters, phosphotriester phosphorothioates, phosphorodithioates, phosphoramidates, alkylphosphonates, alkylphosphonothioates, ketones, sulfones and sulfates.

4. A therapeutic self-stabilized oligonucleotide according to claim 1, wherein the virus is selected from the group consisting of: human immunodeficiency virus, herpes simplex virus, human papilloma virus, influenza virus, foot and mouth disease virus, yellow fever virus, Varicella-Zoster virus, and cucumber mosaic virus.

5. A therapeutic self-stabilized oligonucleotide according to claim 1, wherein the pathogenic organism is selected from the group consisting of Plasmodium falciparum, Trypanosoma brucei, Leishmania, and Fasciola hepatica.

6. A therapeutic self-stabilized oligonucleotide according to claim 1, wherein the cellular gene is selected from the group consisting of prion protein, Alzheimer's amyloid-like protein, and oncogenes or proto-oncogenes.

7. A therapeutic self-stabilized oligonucleotide according to claim 2, wherein the self-complementary region comprises nucleotides selected from the group consisting of: deoxyribonucleotide or ribonucleotide phosphodiesters, phosphorothioates, phosphorodithioates, phosphoramidates, alkylphosphonates, alkylphosphonothioates, phosphoromorpholidates, ketones, sulfones and sulfates.

8. A therapeutic self-stabilized oligonucleotide according to claim 2, wherein the virus is selected from the group consisting of: human immunodeficiency virus, herpes simplex virus, human papilloma virus, influenza virus, foot and mouth disease virus, yellow fever virus, Varicella-Zoster virus, and cucumber mosaic virus.

9. A therapeutic self-stabilized oligonucleotide according to claim 2, wherein the pathogenic organism is selected from the group consisting of Plasmodium falciparum, Trypanosoma brucei, Leishmania, and Fasciola hepatica.

10. A therapeutic self-stabilized oligonucleotide according to claim 2, wherein the cellular gene is

selected from the group consisting of prion protein, Alzheimer's amyloid-like protein, and oncogenes or proto-oncogenes.

11. A therapeutic self-stabilized oligonucleotide according to claim 3, wherein the virus is selected from the group consisting of: human immunodeficiency virus, herpes simplex virus, human papilloma virus, influenza virus, foot and mouth disease virus, yellow fever virus, Varicella-Zoster virus, and cucumber mosaic virus.

12. A therapeutic self-stabilized oligonucleotide according to claim 3, wherein the pathogenic organism is selected from the group consisting of <u>Plasmodium falciparum</u>, <u>Trypanosoma brucei</u>, <u>Leishmania</u>, and <u>Fasciola hepatica</u>.

13. A therapeutic self-stabilized oligonucleotide according to claim 3, wherein the cellular gene is selected from the group consisting of prion protein, Alzheimer's amyloid-like protein, and oncogenes or proto-oncogenes.

14. A self-stabilized ribozyme having a self-complementary region at either or both of its 5' and 3' ends.

15. A method of inhibiting the gene expression of a virus, a pathogenic organism, or a cellular gene, the method comprising providing the self-stabilized oligonucleotide of claim 1 to virus or pathogen infected cells, or to uninfected cells, respectively.

16. A method of inhibiting the gene expression of a virus, a pathogenic organism, or a cellular gene, the method comprising providing the self-stabilized

oligonucleotide of claim 2 to virus or pathogen infected cells, or to uninfected cells, respectively.

17. A method of inhibiting the gene expression of a virus, a pathogenic organism, or a cellular gene, the method comprising providing the self-stabilized oligonucleotide of claim 3 to virus or pathogen infected cells, or to uninfected cells, respectively.

18. A method of treating a diseased human or animal having a disease resulting from a virus or pathogenic organism infection or from the abnormal expression or product of a cellular gene, the method comprising administering to the diseased human or animal oligonucleotide according to claim 1 in a pharmaceutically acceptable carrier.

19. A method of treating a diseased human or animal having a disease resulting from a virus or pathogenic organism infection or from the abnormal expression or product of a cellular gene, the method comprising administering to the diseased human or animal oligonucleotide according to claim 2 in a pharmaceutically acceptable carrier.

20. A method of treating a diseased human or animal having a disease resulting from a virus or pathogenic organism infection or from the abnormal expression or product of a cellular gene, the method comprising administering to the diseased human or animal oligonucleotide according to claim 3 in a pharmaceutically acceptable carrier.

21. A hyperstabilized self-stablized oligonucleotide having one or more ribonucleotide or 2'-O-Me-ribonucleotide in the self-complementary region or in the

complementary portion of the target hybridizing region.

22. A hyperstablized self-stabilized oligonucleotide having one or more intercalating agent molecule in the self-complementary region or in a linker region.

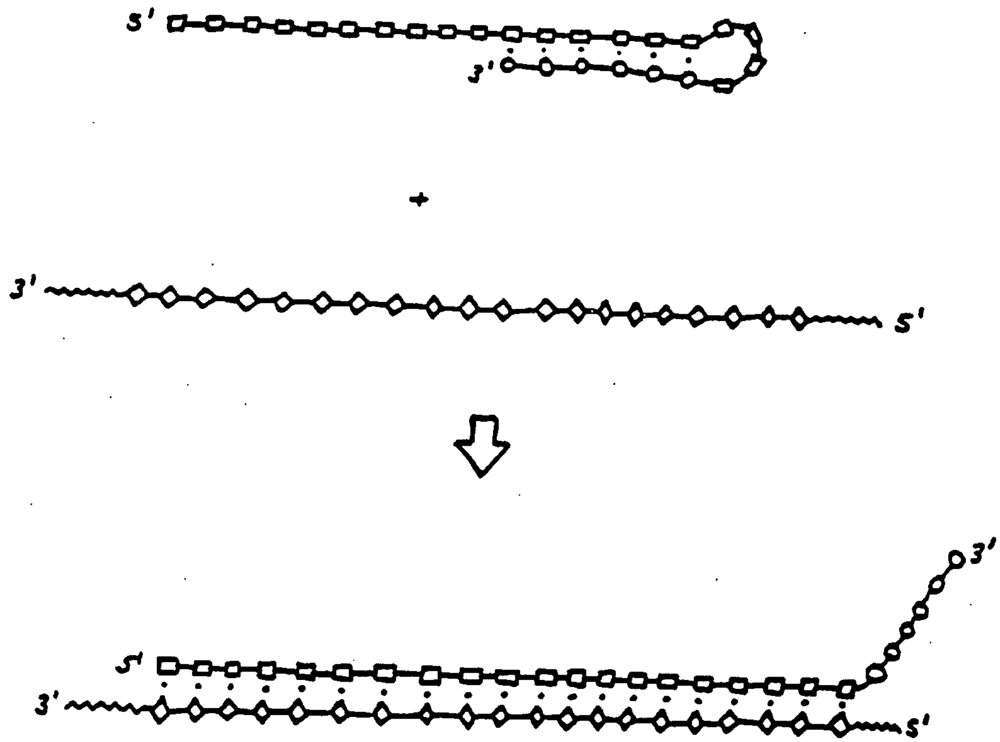


Fig. 1

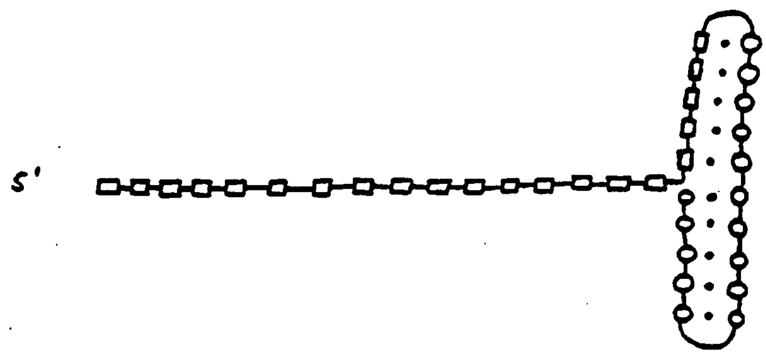


Fig. 2

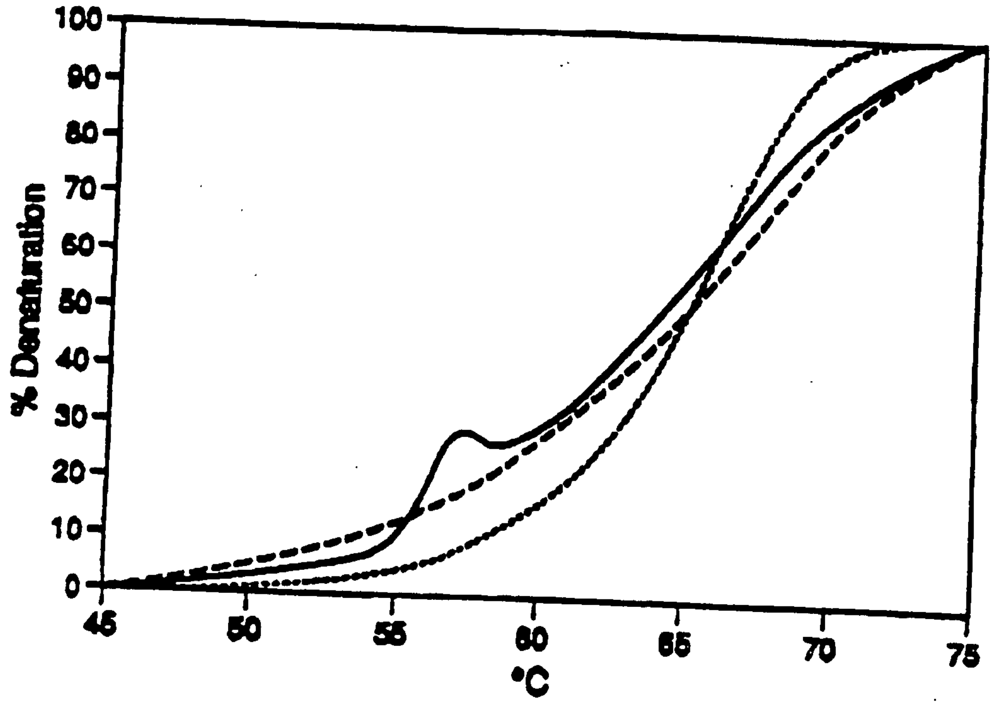


Fig.3

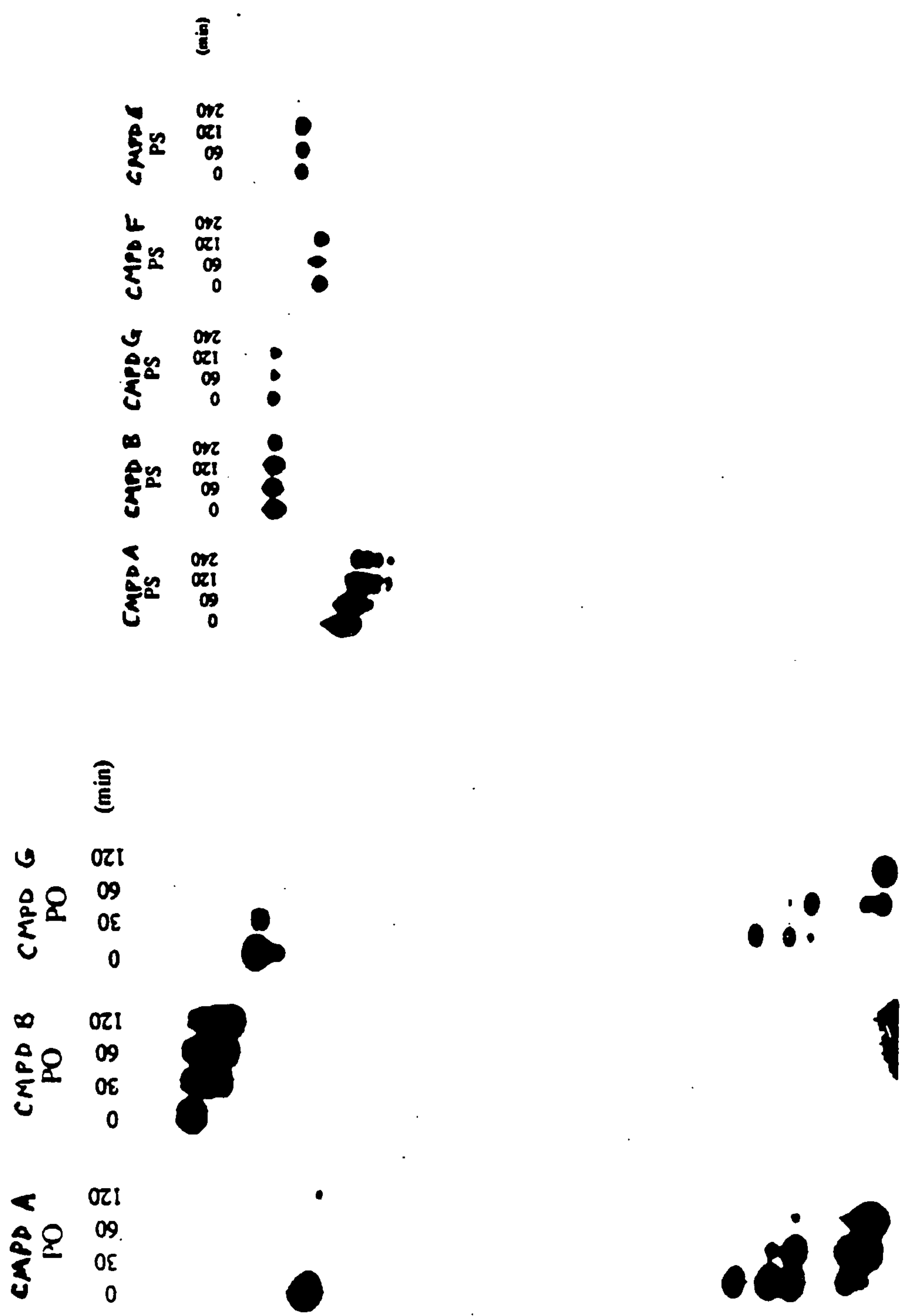

CMPD A PS
CMPD B PS
CMPD G PS
CMPD F PS
CMPD E PS
0 60 120 240
(min)
CMPD A PO
CMPD B PO
CMPD G PO
0 30 60 120
(min)

Fig. 4

CMPD A 5'-CTCTCGCACCCATCTCTCTCCTTCT

CMPD B 5'-CTCTCGCACCCATCTCTCTCC T T
TAGAGAGAGG T C

CMPD C 5'-CTCTCGCACCCA TCTCT C
3'-GAGAGCGTGGGT CTTCC T

CMPD D 5'-CTCTCGCACCCAT CTCTCTCC T T
GAGAGAGG T C

CMPD E 5'-CTCTCGCACCCATCT CTCTCC T T
GAGAGG T C

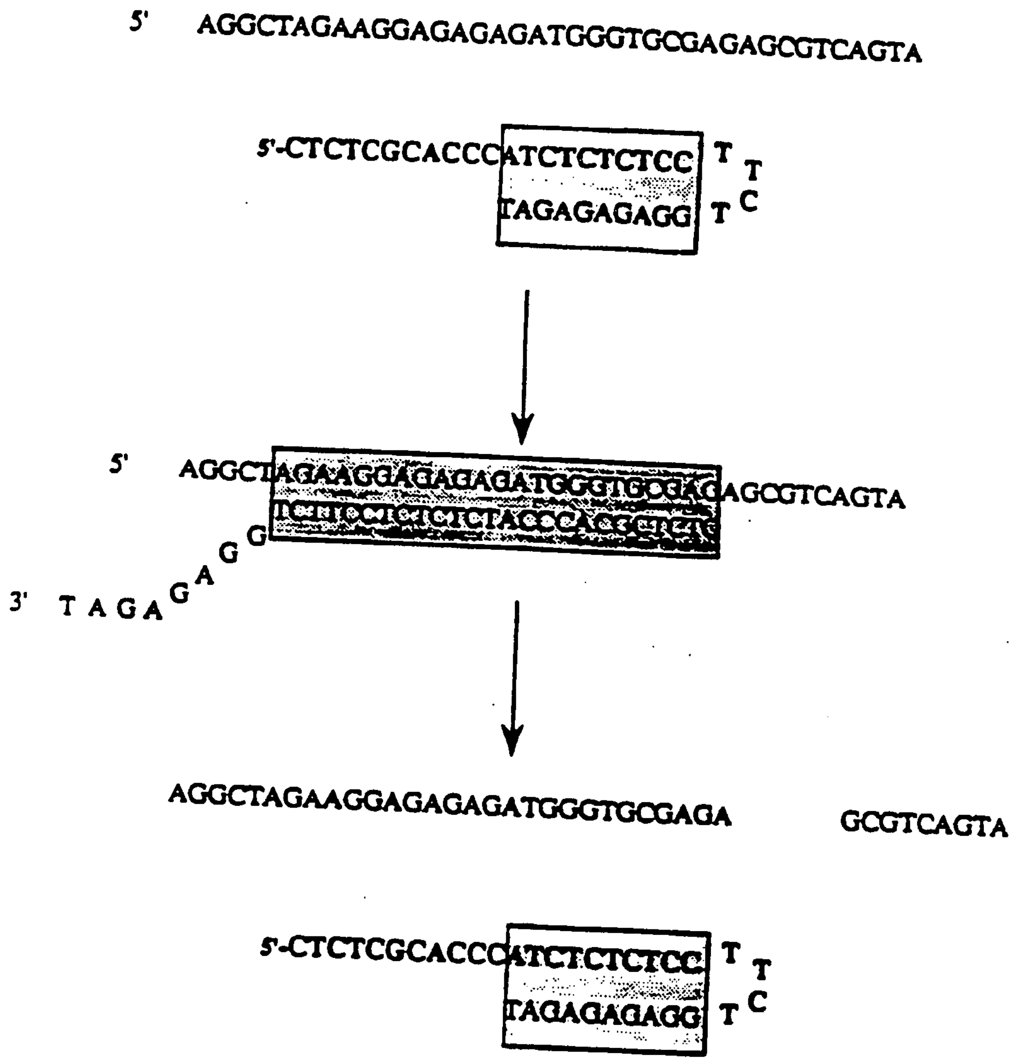
CMPD F 5'-CTCTCGCACCCATCTCT CTCC T T
GAGG T C

CMPD G 5'-CTCTCGCACCCATCTCTCTCC T T
TTTTTTTTTTT T C

CMPD H 5'-CTCTCGCACCCATCTCTCTCCTTCTAGCCTCCGCT 3'

Fig.5

6/7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5'-CTCTCGCACCCATCTCTCTCC T T
TAGAGAGAGG T C
5' AGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTA
3' T A G A G A G G
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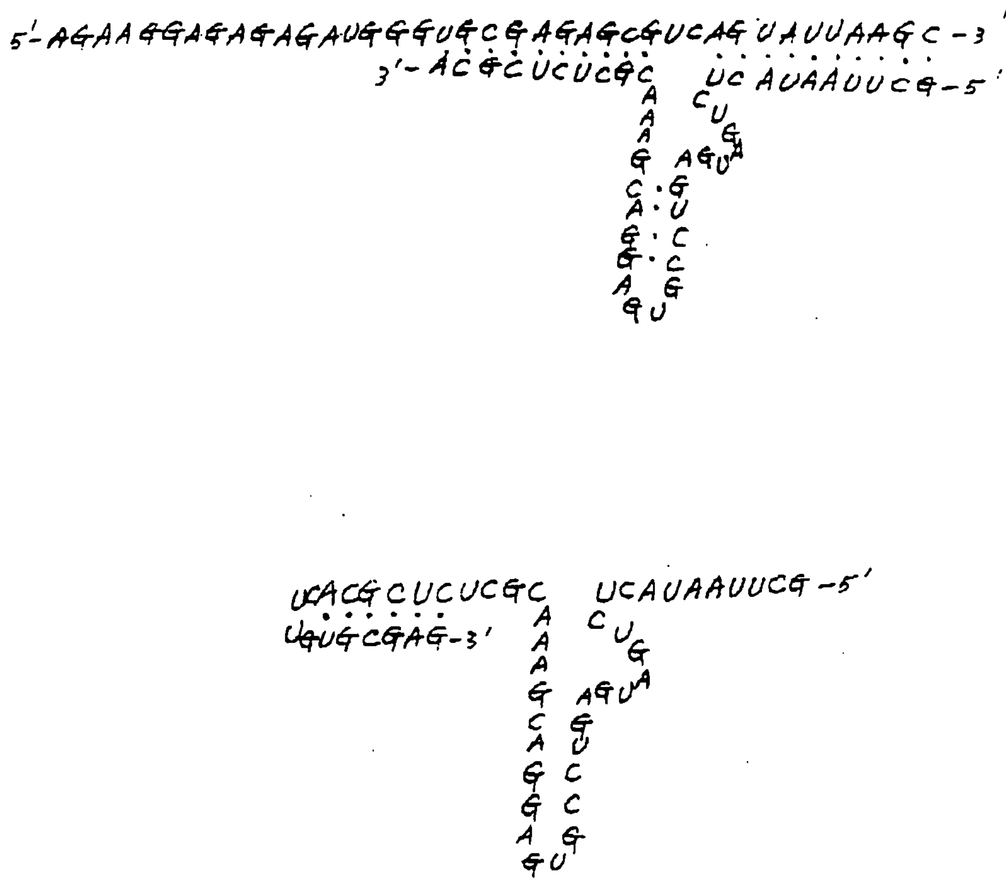
5'-CTCTCGCACCCATCTCTCTCC T T
TAGAGAGAGG T C

Fig. 6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UCACGCUCUCGC UCAUAAUUCG-5'
UGUGCGAG-3'

Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 93/06326

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/11 C07H21/00 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBO JOURNAL vol. 8, no. 13 , 1989 , EYNSHAM, OXFORD GB pages 4297 - 4305 CASE C.C. ET AL 'The unusual stability of the IS10 anti-sense RNA is critical for its function and is determined by the structure of the stem-domain'	1-21
Y	see page 4303, column 2	22
Y	WO,A,92 03464 (MICROPROBE CORPORATION) 5 March 1992 see claims; example 32	22

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
16 November 1993	30. 11. 93

Name and mailing address of the ISA	Authorized officer
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International Application No
PCT/US 93/06326

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	NUCLEIC ACIDS RESEARCH vol. 21, no. 11 , 11 June 1993 , ARLINGTON, VIRGINIA US pages 2729 - 2735 TANG J.Y. ET AL 'Self-stabilized antisense oligodeoxynucleotide phosphorothioates: properties and anti-HIV activity'	1-21
P,Y	see page 2733, column 2, line 20 - page 2735	22

1

INTERNATIONAL SEARCH REPORT	International application No. PCT/US 93/06326

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 18-20 and claims 15-17(partially) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9203464	05-03-92	EP-A- 0547142	23-06-93

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, NZ, PL, RO, RU, SD, SE, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SELF-STABILIZED OLIGONUCLEOTIDES AS THERAPEUTIC AGENTS

(57) Abstract

The invention provides improved antisense oligonucleotides that are resistant to nucleolytic degradation. Such oligonuclotides are called self-stabilized oligonucleotides and comprise two regions: a target hybridizing region having a nucleotide sequence complementary to a nucleic acid sequence that is from a virus, a pathogenic organism, or a cellular gene; and a self-complementary region having an oligonucleotide sequence complementary to a nucleic acid sequence that is within the self-stabilized oligonucleotide.

The target hybridizing region comprises an oligonucleotide sequence that is complementary to a nucleic acid sequence

The self-complementary region comprises an oligonucleotide sequence that is complementary to a nucleic acid sequence

the oligonucleotide forms a totally or partially double-stranded structure

Figure 5 shows the structure of self-stabilized oligonucleotides used in Examples 1-4.

Figure 6 shows a mechanism of therapeutic action of self-stabilized oligonucleotides.

the term oligonucleotide includes polymers of ribonucleotides, deoxyribonucleotides, or both, with ribonucleotide and/or deoxyribonucleotide monomers being connected together via 5' to 3' linkages which may include any of the linkages that are known in the antisense oligonucleotide art.

Oligonucleotides according to the invention are generally characterized by having two regions: a target hybridizing region and a self-complementary region.

the target hybridizing region is from

about 8 to about 50 nucleotides in length.

the term "oligonucleotide sequence that is complementary to a nucleic acid sequence" is intended to mean an oligonucleotide sequence (2 to about 50 nucleotides) that hybridizes to the nucleic acid sequence under physiological conditions, e.g., by Watson-Crick base paring (interaction between oligonucleotide and single-stranded nucleic acid) or by Hoogsteen base pairing. (interaction between oligonucleotide and double-stranded nucleic acid) or by any other means.

The self-complementary region contains oligonucleotide sequences that are complementary to other oligonucleotide sequences within the oligonucleotide. These other oligonucleotide sequences may be within the target hybridizing region

Either the hairpin structure or the hammer-like structure can have loops resulting from non-base-paired nucleotides, as shown in Figure 1 for the hairpin structure,

The number of base-pairs to be formed by intra-molecular hybridization involving the self-complementary region may vary, but should be adequate to maintain a double-stranded structure

the intra-molecular base-pairing can be so extensive as to involve every nucleotide of the oligonucleotide.

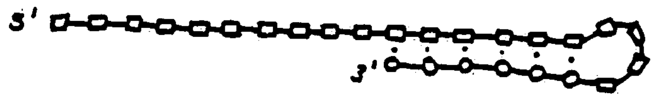

Fig. 1

CMPD C

5'-CTCTCGCACCCA TCTCT C
3'-GAGAGCGTGGGT CTTCC T

Fig.5

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(54) Title: ANTISENSE OLIGONUCLEOTIDE CHEMOTHERAPY FOR BENIGN HYPERPLASIA OR CANCER OF THE PROSTATE

(57) Abstract

Methods of selectively inhibiting the growth of or killing prostatic cells, using antisense oligonucleotides to prostate specific genes, are disclosed. The oligonucleotides may have natural nucleic acid structures or may be modified oligonucleotides with enhanced stability or tissue specific targeting. The prostate specific genes to which the antisense may be directed include the AR and the αFGF gene. Pharmaceutical compositions including such antisense oligonucleotides are also described for use in the methods. The methods and products are of particular utility in the treatment of benign prostatic hyperplasia or prostate cancer.

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ANTISENSE OLIGONUCLEOTIDE CHEMOTHERAPY FOR BENIGN HYPERPLASIA OR CANCER OF THE PROSTATE

Field of the Invention

The present invention relates to the field of chemotherapy for hyperplasias and cancers and, in particular, to chemotherapy for benign hyperplasia or cancer of the prostate. In addition, the invention relates to the field of antisense oligonucleotides and their use in human hyperplasia and cancer therapy.

Background of the Invention

Treatment of carcinoma of the prostate was one of the first successes of cancer chemotherapy, using the therapeutic program of castration and/or anti-androgen hormonal treatments introduced by Charles Huggins in the 1940s. A remarkable relief of symptoms and objective regression of bony metastases occurs under this endocrine therapeutic program. Unfortunately, after a "golden period" which lasts roughly 18 months, regrowth of the prostate cancer cells occurs and, in the later stages of the disease, sensitivity to and repression by anti-androgen hormonal therapy ceases. The conventional regimen of combined chemotherapeutic agents also is typically ineffective after the golden period, and a downhill clinical course follows, terminating in death.

A key problem had been the silent onset of cancer of the prostate, with growth beyond its capsule and metastasis to bone too frequently occurring before the first visit to a physician. During the last half dozen years, there has been increasing recognition of the importance of early diagnosis and significant improvements in the available tests. As a consequence of early diagnosis, detection of prostatic cancer still contained within its capsule has become more frequent. For this situation, radical prostatectomy has largely supplanted the traditional castration/estrogen therapy. Radiation targeted to the prostate itself and to any proximal capsular infiltration has also become a prominent modality of therapy. When these two therapeutic approaches fail to halt progression of the disease, which is all too often (see, e.g., Gittes (1991); and Catalona (1994)), the prospect of benefit from available chemotherapy is gloomy.

Less severe but more common than prostatic cancer is benign prostatic hyperplasia (BPH). This condition may be a precursor to full blown prostatic cancer or may continue for decades without evolving into the deadly carcinoma. Depending upon the degree of hypertrophy

and the age of the patient, treatment may range from "watchful waiting" to more aggressive approaches employing anti-androgen hormonal therapy, transurethral resection, or radical prostatectomy (see, e.g., Catalona (1994)).

The androgen receptor (AR) binds the male hormone testosterone and, acting at the transcriptional level, regulates the growth of normal prostatic cells. A cDNA for the human AR was disclosed by Lubahn et al. (1988). As noted above, anti-androgen or estrogen hormonal therapy, including physical or chemical castration, may be effective against early stage prostate cancer but, after a period of roughly 18 months, the patient becomes refractory to the hormonal therapy. The relapse is believed to be the result of the development or clonal selection of androgen-independent tumor cells in which the AR has mutated or been lost (see, e.g., Taplin, et al. (1995); Klocker, et al. (1994). Interestingly, in murine androgen-independent prostatic cancer cells, transfection with an AR cDNA has been shown to inhibit growth in the presence of testosterone (Suzuki, et al. (1994)).

The acidic fibroblast growth factor (αFGF), also known as the heparin binding growth factor type one (HBGF-1), is an androgen-regulated mitogen produced by prostatic cells. An mRNA sequence for a human allele of αFGF was disclosed in Harris, et al. (1991). Mansson, et al. (1989) found that αFGF was expressed in normal immature rat prostate but not in normal mature rat prostate. In cancerous rat prostatic cell lines, they found αFGF expression similar to that in immature rat prostate.

Summary of the Invention

The present invention provides methods for treating a patient diagnosed as having benign prostatic hyperplasia or a prostatic cancer. The methods include administering to the patient a therapeutically effective amount of a composition comprising an antisense oligonucleotide which selectively hybridizes to an AR or αFGF gene or mRNA sequence of the patient, thereby inhibiting the expression of the AR or αFGF gene or mRNA sequence. This inhibition of the AR or αFGF genes or mRNAs by antisense oligonucleotides results in a significant inhibition of the growth or survival of prostatic cells. As a result, the methods provide a useful new means of treating benign prostatic hyperplasia and prostatic cancer. The methods are particularly useful in treating prostate cancer patients who have become refractory to anti-androgen hormonal therapy.

The AR antisense oligonucleotides may comprise at least 10 consecutive bases from SEQ

ID NO.: 1, at least 10 consecutive bases from a genomic sequence corresponding to SEQ ID NO.: 1, or oligonucleotides that hybridize to the complements of these sequences under physiological conditions. More preferably, the antisense oligonucleotides comprise at least 15 consecutive bases, and most preferably, 20-30 consecutive bases from the above-described sequences.

The αFGF antisense oligonucleotides may comprise at least 10 consecutive bases from any one of SEQ ID NO.: 2, SEQ ID NO.: 3 or SEQ ID NO.: 4, at least 10 consecutive bases from the joined exons of SEQ ID NO.: 2, SEQ ID NO.: 3 and SEQ ID NO.: 4; or oligonucleotides that hybridize to the complements of these sequences under physiological conditions. More preferably, the antisense oligonucleotides comprise at least 15 consecutive bases, and most preferably, 20-30 consecutive bases from the above-described sequences.

Examples of sequences of the invention include, but are not limited to, those disclosed as SEQ ID NO.: 5, SEQ ID NO.: 6, SEQ ID NO.: 7, and SEQ ID NO.: 8.

In preferred embodiments, all of the above-described oligonucleotides are modified oligonucleotides. In one set of embodiments, the modified oligonucleotide includes at least one synthetic internucleoside linkage such as a phosphorothioate, alkylphosphonate, phosphorodithioate, phosphate ester, alkylphosphonothioate, phosphoramidate, carbamate, carbonate, phosphate triester, acetamidate, or carboxymethyl ester.

In other embodiments with modified oligonucleotides, the modified oligonucleotide has at least one low molecular weight organic group covalently bound to a phosphate group of said oligonucleotide. In another set of embodiments, the modified oligonucleotide has at least one low molecular weight organic group covalently bound to a 2' position of a ribose of said oligonucleotide. Such low molecular weight organic groups include lower alkyl chains or aliphatic groups (e.g., methyl, ethyl, propyl, butyl), substituted alkyl and aliphatic groups (e.g., aminoethyl, aminopropyl, aminohydroxyethyl, aminohydroxypropyl), small saccharides or glycosyl groups.

In another set of embodiments the modified oligonucleotide has covalently attached thereto a prostate-targeting compound such as an androgen, androgen derivative, estrogen, estrogen derivative, estramustine, emcyt or estracyt.

In preferred embodiments, the antisense oligonucleotides are administered intravenously at a dosage between 1.0 μg and 100 mg per kg body weight of the patient.

The present invention also provides for any or all of the above-described antisense oligonucleotides, including the various modified oligonucleotides, in a pharmaceutical composition. The antisense oligonucleotides are admixed with a sterile pharmaceutically acceptable carrier in a therapeutically effective amount such that the isolated antisense oligonucleotide selectively hybridizes to the AR or αFGF gene or mRNA sequence when administered to a patient. A pharmaceutical kit is also provided in which such a pharmaceutical composition is combined with a pharmaceutically acceptable carrier for intravenous administration.

The methods and products of the present invention further include antisense oligonucleotides, as described above, directed at a PSA gene, a probasin gene, an estrogen receptor gene, a telomerase gene, a prohibitin gene, a src gene, a ras gene, a myc gene, a blc-2 gene, a protein kinase-A gene, a plasminogen activator urokinase gene and a methyl transferase gene.

Detailed Description of the Invention

The present invention provides new methods for the treatment of cancer of the prostate and pharmaceutical compositions useful therefor. It is now disclosed that antisense oligonucleotides complementary to genes which are expressed predominantly or strongly in prostatic cells are effective for inhibiting the growth of and/or killing hyperplastic or cancerous cells of prostatic origin. In particular, the present invention provides oligonucleotides, including modified oligonucleotides, which have antisense homology to a sufficient portion of either the AR or αFGF gene such that they inhibit the expression of that gene. Surprisingly, inhibition of either of these genes, even in androgen-resistant prostatic cancer cells, inhibits the growth of these cells. Because the antisense oligonucleotides of the invention can be administered systemically but selectively inhibit prostate cells, the present invention has particular utility in late stage prostate cancer which has metastasized.

Definitions

In order to describe more clearly and concisely the subject matter of the present invention, the following definitions are provided for specific terms used in the claims appended hereto:

<u>AR</u>. As used herein, the abbreviation "AR" refers to the androgen receptor well known

in the art and described in the various references cited herein. A cDNA sequence of the human AR gene was disclosed in Lubahn et al. (1988). The Lubahn et al. (1988)sequence is available on GenBank (Accession number J03180) and is reproduced here as SEQ. ID NO.: 1. The translation initiation codon of this gene is found at base positions 363-365 and the stop codon is at positions 3120-3122 of SEQ ID NO.: 1. As will be obvious to one of ordinary skill in the art, other alleles of the AR gene, including other human alleles and homologues from other mammalian species, encoding an AR protein and hybridizing to SEQ ID NO.: 1 under stringent hybridization conditions, will exist in natural populations and are embraced by the term "AR gene" as used herein.

<u>αFGF</u>. As used herein, the term "αFGF" refers to the αFGF protein known in the art and described in the various references cited herein. The genomic DNA of one allele of the human αFGF gene has been partially sequenced and was disclosed in Wang et al. (1989). The Wang et al.(1989) sequences cover the three exons of the αFGF gene as well as some 5', 3' and intron sequences. These sequences are available on GenBank (Accession numbers M23017, M23086 and M23087) and are reproduced here as SEQ. ID NO.: 2, SEQ ID NO.: 3 and SEQ ID NO.: 4. A partial cDNA sequence for a human αFGF gene also may be found in Harris et al. (1991). The locations of the exons are located in the sequence listings. The translation initiation codon is found at positions 602-604 of SEQ ID NO.: 2 and the stop codon is found at positions 496-498. In addition, as will be obvious to one of ordinary skill in the art, other alleles of the αFGF gene, including other human alleles and homologues from other mammalian species, encoding an αFGF protein and hybridizing to one or more of SEQ ID NO.: 2, SEQ ID NO.: 3 or SEQ ID NO.: 4 under stringent hybridization conditions, will exist in natural populations and are embraced by the term "αFGF gene" as used herein.

<u>Antisense Oligonucleotides</u>. As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that mRNA. In particular, by an "AR-antisense oligonucleotide" and by an "αFGF-antisense oligonucleotide" are meant oligonucleotides which hybridize under physiological conditions to the AR gene/mRNA or αFGF gene/mRNA and, thereby, inhibit

transcription/translation of the AR and αFGF genes/mRNAs, respectively. The antisense molecules are designed so as to interfere with transcription or translation of AR or αFGF upon hybridization with the target. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It is preferred that the antisense oligonucleotide be selected so as to hybridize selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions.

Stringent hybridization conditions. As used herein, the term "stringent hybridization conditions" means hybridization conditions from 30°C-60°C and from 5x to 0.1x SSC. Highly stringent hybridization conditions are at 45°C and 0.1x SSC. "Stringent hybridization conditions" is a term of art understood by those of ordinary skill in the art. For any given nucleic acid sequence, stringent hybridization conditions are those conditions of temperature and buffer solution which will permit hybridization of that nucleic acid sequence to its complementary sequence and not to substantially different sequences. The exact conditions which constitute "stringent" conditions, depend upon the length of the nucleic acid sequence and the frequency of occurrence of subsets of that sequence within other non-identical sequences. By varying hybridization conditions from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, one of ordinary skill in the art can, without undue experimentation, determine conditions which will allow a given sequence to hybridize only with identical sequences. Suitable ranges of such stringency conditions are described in Krause, M.H.. and S.A. Aaronson, Methods in Enzymology, 200:546-556 (1991). As used herein with respect to in vivo hybridization conditions, the term "physiological conditions" is considered functionally equivalent to the in vitro stringent hybridization conditions.

I. Design of AR and αFGF Antisense Oligonucleotides

The present invention depends, in part, upon the discovery that the selective inhibition of the expression of AR or αFGF by antisense oligonucleotides in prostatic cells effectively inhibits cell growth and/or causes cell death.

Based upon SEQ ID NO.: 1, SEQ ID NO.: 2, SEQ ID NO.: 3 and SEQ ID NO.: 4, or upon allelic or homologous genomic or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the

present invention. In order to be sufficiently selective and potent for AR or αFGF inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the AR or αFGF mRNA transcripts. Most preferably, the antisense oligonucleotides comprise a complementary sequence of 20-30 bases. Although oligonucleotides may be chosen which are antisense to any region of the AR or αFGF genes or mRNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 3'-untranslated regions or telomerase sites may be targeted. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the AR or αFGF antisense is, preferably, targeted to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al. (1994)) and at which proteins are not expected to bind. Finally, although, SEQ ID NO.: 1 discloses a cDNA sequence and SEQ ID NO.: 2, SEQ ID NO.:3 and SEQ ID NO.: 4 disclose genomic DNA sequences, one of ordinary skill in the art may easily derive the genomic DNA corresponding to the cDNA of SEQ ID NO.: 1 and may easily obtain the cDNA sequence corresponding to SEQ ID NO.: 2, SEQ ID NO.:3 and SEQ ID NO.: 4. Thus, the present invention also provides for antisense oligonucleotides which are complementary to the genomic DNA corresponding to SEQ ID NO.: 1 and the cDNA corresponding to SEQ ID NO.: 2, SEQ ID NO.: 3 and SEQ ID NO.: 4. Similarly, antisense to allelic or homologous cDNAs and genomic DNAs are enabled without undue experimentation.

As will be understood by one of ordinary skill in the art, the antisense oligonucleotides of the present invention need not be perfectly complementary to the AR or αFGF genes or mRNA transcripts in order to be effective. Rather, some degree of mismatches will be acceptable if the antisense oligonucleotide is of sufficient length. In all cases, however, the oligonucleotides should have sufficient length and complementarity so as to hybridize to an AR or αFGF transcript under physiological conditions. Preferably, of course, mismatches are absent or minimal. In addition, although it is not recommended, the antisense oligonucleotides may have one or more non-complementary sequences of bases inserted into an otherwise complementary antisense oligonucleotide sequence. Such non-complementary sequences may "loop" out of a duplex formed by an AR or αFGF transcript and the bases flanking the non-complementary region. Therefore, the entire oligonucleotide may retain an inhibitory effect despite an

apparently low percentage of complementarity. Of particular importance in this respect is the use of self-stabilized or hairpin oligonucleotides. Such oligonucleotides, or modified oligonucleotides, have a sequence at the 5' and/or 3' end which is capable of folding over and forming a duplex with itself. The duplex region, which is preferably at least 4-6 bases joined by a loop of 3-6 bases, stabilizes the oligonucleotide against degradation. These self-stabilized oligonucleotides are easily designed by adding the inverted complement of a 5' or 3' AR or αFGF sequence to the end of the oligonucleotide (see, e.g., Table 1, SEQ ID NO.: 6 and SEQ ID NO.: 7; Tang, J.-Y., et al. (1993) Nucleic Acids Res. 21:2729-2735).

In one set of embodiments, the AR and αFGF antisense oligonucleotides of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one nucleotide and the 3' end of another nucleotide may be covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art recognized methods which may be carried out manually or by an automated synthesizer.

In preferred embodiments, however, the antisense oligonucleotides of the invention also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting to prostatic cells or which otherwise enhance their therapeutic effectiveness. The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide.

Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidate, and carboxymethyl esters. Further, one or more of the 5'→3' phosphate group may be covalently joined to a low molecular weight (e.g., 15-500 Da) organic group. Such low molecular weight organic groups include lower alkyl chains or aliphatic groups (e.g., methyl, ethyl, propyl, butyl), substituted alkyl and aliphatic groups (e.g., aminoethyl, aminopropyl, aminohydroxyethyl, aminohydroxypropyl), small saccharides or glycosyl groups. Other low molecular weight organic modifications include additions to the

internucleoside phosphate linkages such as cholesteryl or diamine compounds with varying numbers of carbon residues between the amino groups and terminal ribose. Oligonucleotides with these linkages or other modifications can be prepared according to known methods (see, e.g., Agrawal and Goodchild (1987); Agrawal et al. (1988); Uhlmann et al. (1990); Agrawal et al. (1992); Agrawal (1993); and U.S. Pat. No. 5,149,798).

The term "modified oligonucleotide" also encompasses oligonucleotides with a covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-alkylated ribose group such as a 2'-O-methylated ribose. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. Alternatively, the modified oligonucleotides may be branched oligonucleotides. Unoxidized or partially oxidized oligonucleotides having a substitution in one or more nonbridging oxygen per nucleotide in the molecule are also considered to be modified oligonucleotides.

Also considered as modified oligonucleotides are oligonucleotides having prostate-targeting, nuclease resistance-conferring, or other bulky substituents and/or various other structural modifications not found *in vivo* without human intervention. The androgen receptor and other hormonal receptor sites on prostate cells allow for targeting antisense oligonucleotides specifically or particularly to prostatic cells. Attachment of the antisense oligonucleotides by a molecular "tether" (e.g., an alkyl chain) to estramustine, emcyt or estracyt (Sheridan and Tew (1991)), for example, may provide prostatic targeting and the possibility of covalent alkylation of host prostatic DNA. Estramustine targets particularly to the ventral prostate (Forsgren, et al. (1979)). Similarly, one may covalently attach androgen, estrogen, androgen or estrogen derivatives, or other prostate cell ligands to antisense oligonucleotides using tethers and conjugating linkages for prostatic targeting. Finally, one may of course covalently attach other chemotherapeutic agents (e.g., dexamethasone, vinblastine, etoposide) to the antisense oligonucleotides for enhanced effect.

The most preferred modified oligonucleotides are hybrid or chimeric oligonucleotides in which some but not all of the phosphodiester linkages, bases or sugars have been modified. Hybrid modified antisense oligonucleotides may be composed, for example, of stretches of ten

2'-O-alkyl nucleotides or ten phosphorothioate synthetic linkages at the 5' and/or 3' ends, and a segment of seven unmodified oligodeoxynucleotides in the center, or of similar terminal segments of alkyl phosphonates, with central P=S or P=O oligonucleotides (Agrawal, et al. (1990); Metelev, et al. (1994)). The currently most preferred modified oligonucleotides are 2'-O-methylated hybrid oligonucleotides. Since degradation occurs mainly at the 3' end, secondarily at the 5' end, and less in the middle, unmodified oligonucleotides located at this position can activate RNase H, and yet are degraded slowly. Furthermore, the T_m of such a 27-mer is approximately 20°C higher than that of a 27-mer all phosphorothioate oligodeoxynucleotide. This greater affinity for the targeted genomic area can result in greater inhibiting efficacy. Obviously, the number of synthetic linkages at the termini need not be ten and synthetic linkages may be combined with other modifications, such as alkylation of a 5' or 3' phosphate, or 2'-O-alkylation. Thus, merely as another example, one may produce a modified oligonucleotide with the following structure, where B represents any base, R is an alkyl, aliphatic or other substituent, the subscript S represents a synthetic (e.g. phosphorothioate) linkage, and each n is an independently chosen integer from 1 to about 20:

$$
\begin{array}{l}
\hphantom{^{5'}(B_S)_nBBBB\text{-} \;\ldots\; \text{-}BBBB(B_S)_nB—}\mathrm{OH} \\
\hphantom{^{5'}(B_S)_nBBBB\text{-} \;\ldots\; \text{-}BBBB(B_S)_nB—}| \\
^{5'}(B_S)_nBBBB\text{-} \;\ldots\; \text{-}BBBB(B_S)_nB—\mathrm{P{=}O}^{3'} \\
\hphantom{^{5'}(B_S)_nBBBB\text{-} \;\ldots\; \text{-}BBBB(B_S)_nB—}| \\
\hphantom{^{5'}(B_S)_nBBBB\text{-} \;\ldots\; \text{-}BBBB(B_S)_nB—}\mathrm{O—R}
\end{array}
$$

II. <u>Products and Methods of Treatment for BPH and Prostate Cancer</u>

The methods of the present invention represent new and useful additions to the field of benign prostate hyperplasia or prostate cancer therapy. In particular, the methods of the present invention are especially useful for late stage prostate cancer in which metastases have occurred and in which the cells have become resistant to estrogen or anti-androgen therapy. The methods may, however, also be used in benign prostate hyperplasia or early stage prostate cancer and may provide a substitute for more radical procedures such as transurethral resection, radical prostatectomy, or physical or chemical castration. The products of the present invention include the isolated antisense oligonucleotides described above. As used herein, the term "isolated" as applied to an antisense oligonucleotide means not covalently bound to and physically separated from the 5' and 3' sequences which flank the corresponding antisense sequence in nature.

Administration of the AR or αFGF antisense oligonucleotides may be oral, intravenous,

parenteral, cutaneous or subcutaneous. For BPH or when the site of a prostatic tumor is known, the administration also may be localized to the prostate or to the region of the tumor by injection to or perfusion of the site.

AR or αFGF antisense oligonucleotides may be administered as part of a pharmaceutical composition. Such a pharmaceutical composition may include the antisense oligonucleotides in combination with any standard physiologically and/or pharmaceutically acceptable carriers which are known in the art. The compositions should be sterile and contain a therapeutically effective amount of the antisense oligonucleotides in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The term "physiologically acceptable" refers to a non-toxic material that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art. The pharmaceutical composition of the invention may also contain other active factors and/or agents which inhibit prostate cell growth or increase cell death. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect or to minimize side-effects caused.

The pharmaceutical composition of the invention may be in the form of a liposome in which the AR or αFGF antisense oligonucleotides are combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers which are in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Pat. No. 4,235,871; U.S. Pat. No. 4,501,728; U.S. Pat. No. 4,837,028; and U.S. Pat. No. 4,737,323.

The pharmaceutical composition of the invention may further include compounds such as cyclodextrins and the like which enhance delivery of oligonucleotides into cells. When the composition is not administered systemically but, rather, is injected at the site of the target cells, cationic detergents (e.g. Lipofectin) may be added to enhance uptake.

When a therapeutically effective amount of AR or αFGF antisense oligonucleotides is administered orally, the oligonucleotides will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder may contain from about 5 to 95% of the AR and/or αFGF antisense oligonucleotides and preferably from about 25 to 90% of the oligonucleotides. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition may contain from about 0.5 to 90% by weight of an AR and/or αFGF antisense oligonucleotide and preferably from about 1 to 50% of the oligonucleotide.

When a therapeutically effective amount of an AR or αFGF antisense oligonucleotide is administered by intravenous, cutaneous or subcutaneous injection, the oligonucleotides will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to the antisense oligonucleotides, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or another vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

In preferred embodiments, when the target cells are readily accessible, administration of the antisense oligonucleotides is localized to the region of the targeted cells in order to maximize the delivery of the antisense and to minimize the amount of antisense needed per treatment. Thus, in one preferred embodiment, administration is by direct injection at or perfusion of the site of the targeted cells, such as a tumor. Alternatively, the antisense oligonucleotides may be adhered to small particles (e.g., microscopic gold beads) which are impelled through the membranes of the target cells (see, e.g., U.S. Pat. No. 5,149,655).

In another series of embodiments, a recombinant gene is constructed which encodes an

AR or αFGF antisense oligonucleotide and this gene is introduced within the targeted cells on a vector. Such an AR or αFGF antisense gene may, for example, consist of the normal AR or αFGF sequence, or a subset of the normal sequences, operably joined in reverse orientation to a promoter region. An operable antisense gene may be introduced on an integration vector or may be introduced on an expression vector. In order to be most effective, it is preferred that the antisense sequences be operably joined to a strong eukaryotic promoter which is inducible or constitutively expressed.

In all of the above-described methods of treatment, the AR and/or αFGF antisense oligonucleotides are administered in therapeutically effective amounts. As used herein, the term "therapeutically effective amount" means that amount of antisense which, under the conditions of administration, including mode of administration and presence of other active components, is sufficient to result in a meaningful patient benefit, i.e., the killing or inhibition of the growth of target cells.

The amount of AR and/or αFGF antisense oligonucleotides in the pharmaceutical composition of the present invention will depend not only upon the potency of the antisense but also upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of antisense with which to treat each individual patient. Initially, the attending physician will administer low doses of the inhibitor and observe the patient's response. Larger doses of antisense may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. In preferred embodiments, it is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 1.0 μg to about 100 mg of oligonucleotide per kg body weight.

The duration of intravenous therapy using the pharmaceutical compositions of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. Because a bolus of oligonucleotides, particularly highly negatively-charged phosphorothioate modified oligonucleotides, may have adverse side effects (e.g., rapid lowering of blood pressure), slow intravenous administration is preferred. Thus, intravenous administration of therapeutically effective amounts over a 12-24 hour period are contemplated. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

The following examples of the use of AR and αFGF antisense are presented merely to illustrate some of the oligonucleotides, including modified oligonucleotides, that may be employed according to the present invention. The particular oligonucleotides used, therefore, should not be construed as limiting of the invention but, rather, as indicative of the wide range of oligonucleotides which may be employed. As will be obvious to one of ordinary skill in the art in light of the present disclosure, a great many equivalents to the presently disclosed antisense oligonucleotides and disclosed methods are now available. In particular, other antisense oligonucleotides substantially complementary to subsets of SEQ ID NO.: 1, SEQ ID NO.: 2, SEQ ID NO.: 3 or SEQ ID NO.: 4 and chemical modifications of the same which do not prevent hybridization under physiological conditions, are contemplated as equivalents of the examples presented below. In general, the use of prostate specific antisense oligonucleotides is contemplated as a method of selectively inhibiting the growth of or killing prostatic cells. In particular, the use of antisense oligonucleotides to the estrogen receptor, PSA, probasin, telomerase, prohibitin, src, ras, myc, blc-2, protein kinase-A, plasminogenctivator urokinase and methyl transferase genes is contemplated for the treatment of benign prostatic hyperplasia or prostatic cancer.

Experimental Examples

The PC3-1435 permanent cell line of human prostatic cancer, obtained from the American Type Culture Collection, was grown in monolayer culture: The PC3-1435 cells are from an osseous metastasis and are androgen-insensitive. Cells were grown in Dulbecco's medium supplemented with 10 percent fetal calf serum, glutamate, pyruvate, penicillin and streptomycin, in 25-150 cm flasks, incubated at 37°C in 6 percent CO_2-air.

A number of AR and αFGF antisense oligonucleotides were tested for their inhibitory effect on prostatic cells. The base sequences of these oligonucleotides are disclosed as SEQ ID NO.: 5 through SEQ ID NO.: 8. SEQ ID NO.: 5 is antisense to positions 927-953 of the AR gene (SEQ ID NO.: 1). SEQ ID NO.: 6 is a self-stabilized or hairpin oligonucleotide. The first 21 bases are complementary to positions 916-936 of the AR gene. The remaining eight are identical to positions 920-927 of the gene, allowing formation of a 3' hairpin. SEQ ID NO.: 7 is another self-stabilized antisense oligonucleotide. The first 21 bases of this oligonucleotide are complementary to positions 927-947 of the AR gene. The remaining eight are identical to

positions 931-938 of the gene, allowing for formation of a 3' hairpin. Finally, SEQ ID NO.: 8 is an antisense sequence corresponding to positions 611-635 of the αFGF gene.

Table 1 shows some of the antisense oligonucleotides tested. The numbers at the left of each sequence correspond to the sequence numbers in the sequence listing. Antisense oligonucleotides with unmodified or natural internucleoside linkages (P=O) and oligonucleotides with all phosphorothioate synthetic linkages (P=S) were tested. In addition, modified oligonucleotides were tested in which just the terminal two phosphodiester linkages at each end had been replaced by phosphorothioate synthetic linkages (shown as a subscript S between nucleotides in Table 1) and/or in which small organic chemical groups (e.g., 2-hydroxy-3-amino-propyl, propylamine) were added to the 3' terminal phosphate or the penultimate 3' phosphate.

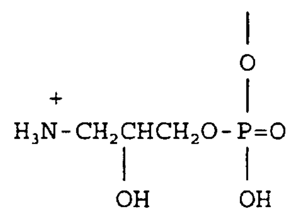
Growth of the PC3-1435 cell line in tissue culture monolayers was consistently inhibited by addition of phosphorothioate-modified oligodeoxynucleotides targeted against the AR or αFGF genes and incubation for 24-48 hours thereafter. As the concentration of modified oligonucleotides is decreased from the 10-20 μM level, most effective inhibition occurs with specific antisense oligodeoxynucleotides at the 2-5 μM level, as contrasted with mismatched oligodeoxynucleotides (see Tables 2 and 3).

While the effects on cell growth (i.e. cell numbers) are readily manifest, visual substage microscopy of wells revealed additional features of the inhibition events using AR antisense oligonucleotides against PC3-1435 cells. The first evidence of antisense inhibition is rupture of the monolayer fabric. The stellate cells in a confluent culture lose contact with their neighbors, round up individually or in clumps, become pyknotic, and cease growing, as examined on successive days. There is an early loss of adhesiveness to the floor of the plastic wells. These changes are more severe (see Table 4) than those measured by ^{3}H-thymidine incorporation into DNA, in other words more drastic than the impairment of DNA synthesis.

Each of the above-mentioned references and patents are incorporated by reference.

TABLE 1

Antisense Oligonucleotides

	Sequence	Target
#5	$^{5'}$CTG-CTG-CTG-TTG-CTG-AAG-GAG-TTG-CAT$^{3'}$	Androgen receptor, P=S
#5	$^{5'}$CTG-CTG-CTG-TTG-CTG-AAG-GAG-TTG-CAT$^{3'}$	Androgen receptor, P=O
#5	$^{5'}C_ST_SG$-CTG-CTG-TTG-CTG-AAG-GAG-TTG-$C_SA_ST^{3'}$	Androgen receptor, P=S termini
#5	$^{5'}$CTG-CTG-CTG-TTG-CTG-AAG-GAG-TTG-CAT$^{3'}$ 	Androgen receptor, modified with organic group
#5	$^{5'}$CTG-CTG-CTG-TTG-CTG-AAG-GAG-TTG-CA-O-P(=O)(NHCH$_2$CH$_2$CH$_3$)-O-T$^{3'}$	Androgen receptor, modified with organic group
#6	$^{5'}$GGA-GTT-GCA-TGG-TGC-TGG-CCT-CAG-CAC-CA$^{3'}$	Androgen receptor 3' hairpin, P=S
#7	$^{5'}$CTG-TTG-CTG-AAG-GAG-TTG-CAT-AAC-TCC-TT$^{3'}$	Androgen receptor 3' hairpin, P=S
#8	$^{5'}$GGG-CTG-TGA-AGG-TGG-TGA-TTT-CCC-C$^{3'}$	αFGF, P=S

#8	5'GGG-CTG-TGA-AGG-TGG-TGA-TTT-CCC-C3'	αFGF, P=O

TABLE 2

^{3}H-thymidine incorporation into DNA PC3-1435 human prostate cancer tissue culture

Genes Targeted	Concentration (μM)	CPM†	% inhibition
Control (no oligo)	--	38,000	0
Androgen receptor, (P = S)	20	15,000	60
	5	20,000	48
Androgen receptor, (P = S)*	20	10,200	68
	5	24,000	25
Mismatch (P = S)	20	20,000	47
	5	27,000	30

† Averages of 3 separate wells

* 3' phosphate modified with $-CH_2CHOHCHNH_3^+$

TABLE 3

Degree of inhibition of DNA synthesis in PC3-1435 prostate cancer tissue cultures

Genes targeted	Concentration (μM)	CPM †	% inhibition
Control (no oligo)	--	14,700	0
αFGF (P=S)	20	2,485	83
	5	4,500	69
Mismatch	20	6,990	51
	5	10,750	27

† Averages of 3 separate wells.

TABLE 4

Morphological Comparison of Treated and Control Cells

	Concentration µM			
Gene Target	20	10	5	2
αFGF (P=S)	4+	4+	1-1/2+	1+
Androgen receptor (P=S)	3+	3+	1+	1+
Mismatch (P=S)	1-1/2+	1/2+	0	0

Observation 24 hours after oligonucleotide addition. Damage: 4+ devastating; 3+ severe; 2+ serious; 1+ visible; 1/2+ slight; 0 none

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: WORCESTER FOUNDATION FOR BIOMEDICAL RESEARCH, INC.

(ii) TITLE OF INVENTION: ANTISENSE OLIGONUCLEOTIDE CHEMOTHERAPY FOR BENIGN HYPERPLASIA OR CANCER OF THE PROSTATE

(iii) NUMBER OF SEQUENCES: 8

(iv) CORRESPONDENCE ADDRESS:
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(E) COUNTRY: USA
(F) ZIP: 02210

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: TWOMEY, MICHAEL J.
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(C) REFERENCE/DOCKET NUMBER: W0461/7035

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3569 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 363..3122

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAATAACTCA GTTCTTATTT GCACCTACTT CAGTGGACAC TGAATTTGGA AGGTGGAGGA 60

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TTTTGTTTTT TTCTTTTAAG ATCTGGGCAT CTTTTGAATC TACCCTTCAA GTATTAAGAG  120

ACAGACTGTG AGCCTAGCAG GGCAGATCTT GTCCACCGTG TGTCTTCTTC TGCACGAGAC  180

TTTGAGGCTG TCAGAGCGCT TTTTGCGTGG TTGCTCCCGC AAGTTTCCTT CTCTGGAGCT  240

TCCCGCAGGT GGGCAGCTAG CTGCAGCGAC TACCGCATCA TCACAGCCTG TTGAACTCTT  300

CTGAGCAAGA GAAGGGGAGG CGGGGTAAGG GAAGTAGGTG GAAGATTCAG CCAAGCTCAA  360

GG ATG GAA GTG CAG TTA GGG CTG GGA AGG GTC TAC CCT CGG CCG CCG     407
   Met Glu Val Gln Leu Gly Leu Gly Arg Val Tyr Pro Arg Pro Pro
     1               5                  10                  15

TCC AAG ACC TAC CGA GGA GCT TTC CAG AAT CTG TTC CAG AGC GTG CGC    455
Ser Lys Thr Tyr Arg Gly Ala Phe Gln Asn Leu Phe Gln Ser Val Arg
                 20                  25                  30

GAA GTG ATC CAG AAC CCG GGC CCC AGG CAC CCA GAG GCC GCG AGC GCA    503
Glu Val Ile Gln Asn Pro Gly Pro Arg His Pro Glu Ala Ala Ser Ala
             35                  40                  45

GCA CCT CCC GGC GCC AGT TTG CTG CTG CTG CAG CAG CAG CAG CAG CAG    551
Ala Pro Pro Gly Ala Ser Leu Leu Leu Leu Gln Gln Gln Gln Gln Gln
         50                  55                  60
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CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAA GAG     599
Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Glu
    65                      70                      75

ACT AGC CCC AGG CAG CAG CAG CAG CAG CAG GGT GAG GAT GGT TCT CCC     647
Thr Ser Pro Arg Gln Gln Gln Gln Gln Gln Gly Glu Asp Gly Ser Pro
 80                     85                      90                  95

CAA GCC CAT CGT AGA GGC CCC ACA GGC TAC CTG GTC CTG GAT GAG GAA     695
Gln Ala His Arg Arg Gly Pro Thr Gly Tyr Leu Val Leu Asp Glu Glu
                100                     105                     110

CAG CAA CCT TCA CAG CCG CAG TCG GCC CTG GAG TGC CAC CCC GAG AGA     743
Gln Gln Pro Ser Gln Pro Gln Ser Ala Leu Glu Cys His Pro Glu Arg
            115                     120                     125

GGT TGC GTC CCA GAG CCT GGA GCC GCC GTG GCC GCC AGC AAG GGG CTG     791
Gly Cys Val Pro Glu Pro Gly Ala Ala Val Ala Ala Ser Lys Gly Leu
        130                     135                     140

CCG CAG CAG CTG CCA GCA CCT CCG GAC GAG GAT GAC TCA GCT GCC CCA     839
Pro Gln Gln Leu Pro Ala Pro Pro Asp Glu Asp Asp Ser Ala Ala Pro
    145                     150                     155

TCC ACG TTG TCC CTG CTG GGC CCC ACT TTC CCC GGC TTA AGC AGC TGC     887
Ser Thr Leu Ser Leu Leu Gly Pro Thr Phe Pro Gly Leu Ser Ser Cys
160                     165                     170                     175

TCC GCT GAC CTT AAA GAC ATC CTG AGC GAG GCC AGC ACC ATG CAA CTC     935
Ser Ala Asp Leu Lys Asp Ile Leu Ser Glu Ala Ser Thr Met Gln Leu
                180                     185                     190

CTT CAG CAA CAG CAG CAG GAA GCA GTA TCC GAA GGC AGC AGC AGC GGG     983
Leu Gln Gln Gln Gln Gln Glu Ala Val Ser Glu Gly Ser Ser Ser Gly
            195                     200                     205
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AGA GCG AGG GAG GCC TCG GGG GCT CCC ACT TCC TCC AAG GAC AAT TAC    1031
Arg Ala Arg Glu Ala Ser Gly Ala Pro Thr Ser Ser Lys Asp Asn Tyr
        210                 215                 220

TTA GGG GGC ACT TCG ACC ATT TCT GAC AAC GCC AAG GAG TTG TGT AAG    1079
Leu Gly Gly Thr Ser Thr Ile Ser Asp Asn Ala Lys Glu Leu Cys Lys
    225                 230                 235

GCA GTG TCG GTG TCC ATG GGC CTG GGT GTG GAG GCG TTG GAG CAT CTG    1127
Ala Val Ser Val Ser Met Gly Leu Gly Val Glu Ala Leu Glu His Leu
240                 245                 250                 255

AGT CCA GGG GAA CAG CTT CGG GGG GAT TGC ATG TAC GCC CCA CTT TTG    1175
Ser Pro Gly Glu Gln Leu Arg Gly Asp Cys Met Tyr Ala Pro Leu Leu
                260                 265                 270

GGA GTT CCA CCC GCT GTG CGT CCC ACT CCT TGT GCC CCA TTG GCC GAA    1223
Gly Val Pro Pro Ala Val Arg Pro Thr Pro Cys Ala Pro Leu Ala Glu
            275                 280                 285

TGC AAA GGT TCT CTG CTA GAC GAC AGC GCA GGC AAG AGC ACT GAA GAT    1271
Cys Lys Gly Ser Leu Leu Asp Asp Ser Ala Gly Lys Ser Thr Glu Asp
        290                 295                 300

ACT GCT GAG TAT TCC CCT TTC AAG GGA GGT TAC ACC AAA GGG CTA GAA    1319
Thr Ala Glu Tyr Ser Pro Phe Lys Gly Gly Tyr Thr Lys Gly Leu Glu
    305                 310                 315

GGC GAG AGC CTA GGC TGC TCT GGC AGC GCT GCA GCA GGG AGC TCC GGG    1367
Gly Glu Ser Leu Gly Cys Ser Gly Ser Ala Ala Ala Gly Ser Ser Gly
320                 325                 330                 335

ACA CTT GAA CTG CCG TCT ACC CTG TCT CTC TAC AAG TCC GGA GCA CTG    1415
Thr Leu Glu Leu Pro Ser Thr Leu Ser Leu Tyr Lys Ser Gly Ala Leu
                340                 345                 350
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GAC GAG GCA GCT GCG TAC CAG AGT CGC GAC TAC TAC AAC TTT CCA CTG    1463
Asp Glu Ala Ala Ala Tyr Gln Ser Arg Asp Tyr Tyr Asn Phe Pro Leu
            355                 360                 365

GCT CTG GCC GGA CCG CCG CCC CCT CCG CCG CCT CCC CAT CCC CAC GCT    1511
Ala Leu Ala Gly Pro Pro Pro Pro Pro Pro Pro Pro His Pro His Ala
        370                 375                 380

CGC ATC AAG CTG GAG AAC CCG CTG GAC TAC GGC AGC GCC TGG GCG GCT    1559
Arg Ile Lys Leu Glu Asn Pro Leu Asp Tyr Gly Ser Ala Trp Ala Ala
    385                 390                 395

GCG GCG GCG CAG TGC CGC TAT GGG GAC CTG GCG AGC CTG CAT GGC GCG    1607
Ala Ala Ala Gln Cys Arg Tyr Gly Asp Leu Ala Ser Leu His Gly Ala
400                 405                 410                 415

GGT GCA GCG GGA CCC GGT TCT GGG TCA CCC TCA GCC GCC GCT TCC TCA    1655
Gly Ala Ala Gly Pro Gly Ser Gly Ser Pro Ser Ala Ala Ala Ser Ser
                420                 425                 430

TCC TGG CAC ACT CTC TTC ACA GCC GAA GAA GGC CAG TTG TAT GGA CCG    1703
Ser Trp His Thr Leu Phe Thr Ala Glu Glu Gly Gln Leu Tyr Gly Pro
            435                 440                 445

TGT GGT GGT GGT GGG GGT GGT GGC GGC GGC GGC GGC GGC GGC GGC GGC    1751
Cys Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly Gly
        450                 455                 460

GGC GGC GGC GGC GGC GGC GGC GGC GGC GAG GCG GGA GCT GTA GCC CCC    1799
Gly Gly Gly Gly Gly Gly Gly Gly Gly Glu Ala Gly Ala Val Ala Pro
    465                 470                 475

TAC GGC TAC ACT CGG CCC CCT CAG GGG CTG GCG GGC CAG GAA AGC GAC    1847
Tyr Gly Tyr Thr Arg Pro Pro Gln Gly Leu Ala Gly Gln Glu Ser Asp
480                 485                 490                 495
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TTC ACC GCA CCT GAT GTG TGG TAC CCT GGC GGC ATG GTG AGC AGA GTG    1895
Phe Thr Ala Pro Asp Val Trp Tyr Pro Gly Gly Met Val Ser Arg Val
                500                 505                 510

CCC TAT CCC AGT CCC ACT TGT GTC AAA AGC GAA ATG GGC CCC TGG ATG    1943
Pro Tyr Pro Ser Pro Thr Cys Val Lys Ser Glu Met Gly Pro Trp Met
            515                 520                 525

GAT AGC TAC TCC GGA CCT TAC GGG GAC ATG CGT TTG GAG ACT GCC AGG    1991
Asp Ser Tyr Ser Gly Pro Tyr Gly Asp Met Arg Leu Glu Thr Ala Arg
        530                 535                 540

GAC CAT GTT TTG CCC ATT GAC TAT TAC TTT CCA CCC CAG AAG ACC TGC    2039
Asp His Val Leu Pro Ile Asp Tyr Tyr Phe Pro Pro Gln Lys Thr Cys
    545                 550                 555

CTG ATC TGT GGA GAT GAA GCT TCT GGG TGT CAC TAT GGA GCT CTC ACA    2087
Leu Ile Cys Gly Asp Glu Ala Ser Gly Cys His Tyr Gly Ala Leu Thr
560                 565                 570                 575

TGT GGA AGC TGC AAG GTC TTC TTC AAA AGA GCC GCT GAA GGG AAA CAG    2135
Cys Gly Ser Cys Lys Val Phe Phe Lys Arg Ala Ala Glu Gly Lys Gln
                580                 585                 590

AAG TAC CTG TGC GCC AGC AGA AAT GAT TGC ACT ATT GAT AAA TTC CGA    2183
Lys Tyr Leu Cys Ala Ser Arg Asn Asp Cys Thr Ile Asp Lys Phe Arg
            595                 600                 605

AGG AAA AAT TGT CCA TCT TGT CGT CTT CGG AAA TGT TAT GAA GCA GGG    2231
Arg Lys Asn Cys Pro Ser Cys Arg Leu Arg Lys Cys Tyr Glu Ala Gly
        610                 615                 620

ATG ACT CTG GGA GCC CGG AAG CTG AAG AAA CTT GGT AAT CTG AAA CTA    2279
Met Thr Leu Gly Ala Arg Lys Leu Lys Lys Leu Gly Asn Leu Lys Leu
    625                 630                 635
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CAG GAG GAA GGA GAG GCT TCC AGC ACC ACC AGC CCC ACT GAG GAG ACA 2327
Gln Glu Glu Gly Glu Ala Ser Ser Thr Thr Ser Pro Thr Glu Glu Thr
640 645 650 655

ACC CAG AAG CTG ACA GTG TCA CAC ATT GAA GGC TAT GAA TGT CAG CCC 2375
Thr Gln Lys Leu Thr Val Ser His Ile Glu Gly Tyr Glu Cys Gln Pro
660 665 670

ATC TTT CTG AAT GTC CTG GAA GCC ATT GAG CCA GGT GTA GTG TGT GCT 2423
Ile Phe Leu Asn Val Leu Glu Ala Ile Glu Pro Gly Val Val Cys Ala
675 680 685

GGA CAC GAC AAC AAC CAG CCC GAC TCC TTT GCA GCC TTG CTC TCT AGC 2471
Gly His Asp Asn Asn Gln Pro Asp Ser Phe Ala Ala Leu Leu Ser Ser
690 695 700

CTC AAT GAA CTG GGA GAG AGA CAG CTT GTA CAC GTG GTC AAG TGG GCC 2519
Leu Asn Glu Leu Gly Glu Arg Gln Leu Val His Val Val Lys Trp Ala
705 710 715

AAG GCC TTG CCT GGC TTC CGC AAC TTA CAC GTG GAC GAC CAG ATG GCT 2567
Lys Ala Leu Pro Gly Phe Arg Asn Leu His Val Asp Asp Gln Met Ala
720 725 730 735

GTC ATT CAG TAC TCC TGG ATG GGG CTC ATG GTG TTT GCC ATG GGC TGG 2615
Val Ile Gln Tyr Ser Trp Met Gly Leu Met Val Phe Ala Met Gly Trp
740 745 750

CGA TCC TTC ACC AAT GTC AAC TCC AGG ATG CTC TAC TTC GCC CCT GAT 2663
Arg Ser Phe Thr Asn Val Asn Ser Arg Met Leu Tyr Phe Ala Pro Asp
755 760 765

CTG GTT TTC AAT GAG TAC CGC ATG CAC AAG TCC CGG ATG TAC AGC CAG 2711
Leu Val Phe Asn Glu Tyr Arg Met His Lys Ser Arg Met Tyr Ser Gln
770 775 780

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TGT GTC CGA ATG AGG CAC CTC TCT CAA GAG TTT GGA TGG CTC CAA ATC    2759
Cys Val Arg Met Arg His Leu Ser Gln Glu Phe Gly Trp Leu Gln Ile
    785                 790                 795

ACC CCC CAG GAA TTC CTG TGC ATG AAA GCA CTG CTA CTC TTC AGC ATT    2807
Thr Pro Gln Glu Phe Leu Cys Met Lys Ala Leu Leu Leu Phe Ser Ile
800                 805                 810                 815

ATT CCA GTG GAT GGG CTG AAA AAT CAA AAA TTC TTT GAT GAA CTT CGA    2855
Ile Pro Val Asp Gly Leu Lys Asn Gln Lys Phe Phe Asp Glu Leu Arg
                820                 825                 830

ATG AAC TAC ATC AAG GAA CTC GAT CGT ATC ATT GCA TGC AAA AGA AAA    2903
Met Asn Tyr Ile Lys Glu Leu Asp Arg Ile Ile Ala Cys Lys Arg Lys
            835                 840                 845

AAT CCC ACA TCC TGC TCA AGA CGC TTC TAC CAG CTC ACC AAG CTC CTG    2951
Asn Pro Thr Ser Cys Ser Arg Arg Phe Tyr Gln Leu Thr Lys Leu Leu
        850                 855                 860

GAC TCC GTG CAG CCT ATT GCG AGA GAG CTG CAT CAG TTC ACT TTT GAC    2999
Asp Ser Val Gln Pro Ile Ala Arg Glu Leu His Gln Phe Thr Phe Asp
    865                 870                 875

CTG CTA ATC AAG TCA CAC ATG GTG AGC GTG GAC TTT CCG GAA ATG ATG    3047
Leu Leu Ile Lys Ser His Met Val Ser Val Asp Phe Pro Glu Met Met
880                 885                 890                 895

GCA GAG ATC ATC TCT GTG CAA GTG CCC AAG ATC CTT TCT GGG AAA GTC    3095
Ala Glu Ile Ile Ser Val Gln Val Pro Lys Ile Leu Ser Gly Lys Val
                900                 905                 910

AAG CCC ATC TAT TTC CAC ACC CAG TGAAGCATTG GAAACCCTAT TTCCCCACCC  3149
Lys Pro Ile Tyr Phe His Thr Gln
            915                 920
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CAGCTCATGC CCCCTTTCAG ATGTCTTCTG CCTGTTATAA CTCTGCACTA CTCCTCTGCA 3209

GTGCCTTGGG GAATTTCCTC TATTGATGTA CAGTCTGTCA TGAACATGTT CCTGAATTCT 3269

ATTTGCTGGG CTTTTTTTTT CTCTTTCTCT CCTTTCTTTT TCTTCTTCCC TCCCTATCTA 3329

ACCCTCCCAT GGCACCTTCA GACTTTGCTT CCCATTGTGG CTCCTATCTG TGTTTTGAAT 3389

GGTGTTGTAT GCCTTTAAAT CTGTGATGAT CCTCATATGG CCCAGTGTCA AGTTGTGCTT 3449

GTTTACAGCA CTACTCTGTG CCAGCCACAC AAACGTTTAC TTATCTTATG CCACGGGAAG 3509

TTTAGAGAGC TAAGATTATC TGGGGAAATC AAAACAAAAA ACAAGCAAAC AAAAAAAAAA 3569

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1082 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 602..770

(D) OTHER INFORMATION: /note= "SEGMENT 1 OF 3."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAGCTTCCCT TAACATACTA ACCCTTTACT TTCCCTGTTG TGTCCCTGAA AGGCCTCCTG 60

TGCCTTTGGC TGCAGGTCCC GAACGTCCAG GCCATCTGTG CTATCTGCTT CGCGGTACCT 120

CACCAACGCA ACGTGAGGGT GGAGGGCAGA ACCTTGGTCC TGGCCTCTCA GCTTTTGTGG 180

GTTTCAGCCA GACCCTAGGT GTTATTTTAG TGCAACTTTG GTGTTTAATT TGAGGATGTG 240

TGTGGACCAG AAGGAGGGAC CAAAACATGA TTCTTTTCCC CATGGTCAGA TGATTAAATT 300

TGAAGTTCTA AAAAATGCAG TTTGGTCCAA AGCTGTGTCC AATTGGGAAG AGAGAAAAAT 360

GCCCTGGAAA CCCCTCCCAG GCCTGGGACC ATCCTTCCTT AACCACCAGC CACCTCACAG 420

GCCCGCGGAC TGCGGGCATC ACCTGGGCAG GCTGTGCTTA CTCACTACCC GGGAACCCTG 480

TGCCCTGGAG CTGTCCTTCC TCTCTTCAAA GTGCATTTTG TGCCTTTGCT GGAAGAACCG 540

ACTACAGGTT TGTTCAATTT CTTACAGTCT TGAAAGCGCC ACAAGCAGCA GCTGCTGAGC 600

CATGGCTGAA GGGGAAATCA CCACCTTCAC AGCCCTGACC GAGAAGTTTA ATCTGCCTCC 660

AGGGAATTAC AAGAAGCCCA AACTCCTCTA CTGTAGCAAC GGGGGCCACT TCCTGAGGAT 720

CCTTCCGGAT GGCACAGTGG ATGGGACAAG GGACAGGAGC GACCAGCACA GTAAGCCCAT 780

CTCTATGGCA CCCCCCTTCC CTTTCTGACA TCTTCTGTAG TCAAGGTGGG AGGAAGGTGC 840

ACATTTAAGT ACAGGTACTT GCTTCTCCAA GGTTCTATTC AGGCATGACA CATTCAGAGG 900

TGGAGTCACA TAAATGCGTA AAATGTCTGG GAAATGAAAA TAGGGACTTG TGGGGGCCAC 960

CACTTACCCA AACGTGTCCT ATTTCAAGTT TTTTAAAGCA CTCTCTGCTG ACCCAACAGA 1020

ACGGGCTGCC GGTGCTCAAT TGCTGTATGT TTTCCCAGGT TTCTGTAACT AGTGAAAGAT 1080

CT 1082

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 427 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 186..289
(D) OTHER INFORMATION: /note= "SEGMENT 2 OF 3. UNKNOWN NUMBER OF BP AFTER SEGMENT 1."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAGCTTTCTT TGGAAGGCAA AGAAAAAGGG ACTGTATTTC TATGTTTTGA TTAATCTGAG 60

GCTCATCCTG AGGGCTCCGT GAAATGAATG AGCAGAATTT TCCATGGCCA ACTGTCCTGG 120

CTGCCGGGTC CTATCGGCAA AAGCGTAGTG TTTATTTACT TTTGCTCGTG TTATTTTTAT 180

TCCAGTTCAG CTGCAGCTCA GTGCGGAAAG CGTGGGGGAG GTGTATATAA AGAGTACCGA 240

GACTGGCCAG TACTTGGCCA TGGACACCGA CGGGCTTTTA TACGGCTCAG TAAGTATGAA 300

GCTGACATGC TTCCAGACGT TGGCCAAGGT TTGAGGTTTC CAGAAATCTT GTTACATGGA 360

GTGAGGCAAA CTATAAAGCA ACAATTAGTC TCTGTTTGTT ATTTTTTCCA GAAGGATTCC 420

CACCCTC 427

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 664 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION: 304..498

(D) OTHER INFORMATION: /note= "SEGMENT 3 OF 3. UNKNOWN NUMBER OF BP AFTER SEGMENT 2."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGAGGACTCT TAGAAGTGCT CTTATCAGTA GCATCTTAAT TACTTTACAA TGGATTTTAA 60

ATGGAAAGGA AGTTTACAAT AATAGCAAAT GCATATTGAC AGCTCTTTAG TGCCCGGTGC 120

TGTTCTAAGT CCTTATGACT ACCCTGTGAA ATAAGTTCCA CCATGACCCC AATTTTCCTG 180

AAAAGGAGAC TGAGGCATGG AGAGCTTTAG TATTTTGCCC AATGTCACAC AGCTAGTAAA 240

TGGGGACCCC CATGTGAAAC TACTCACTGA TTGTCCTACT CTCTTGTGGT TTTATCTTTT 300

TAGCAGACAC CAAATGAGGA ATGTTTGTTC CTGGAAAGGC TGGAGGAGAA CCATTACAAC 360

ACCTATATAT CCAAGAAGCA TGCAGAGAAG AATTGGTTTG TTGGCCTCAA GAAGAATGGG 420

AGCTGCAAAC GCGGTCCTCG GACTCACTAT GGCCAGAAAG CAATCTTGTT TCTCCCCCTG 480

CCAGTCTCTT CTGATTAAAG AGATCTGTTC TGGGTGTTGA CCACTCCAGA GAAGTTTCGA 540

GGGGTCCTCA CCTGGTTGAC CCAAAAATGT TCCCTTGACC ATTGGCTGCG CTAACCCCCA 600

GCCCACAGAG CCTGAATTTG TAAGCAACTT GCTTCTAAAT GCCCAGTTCA CTTCTTTGCA 660

GAGC 664

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: SYNTHETIC OLIGONUCLEOTIDE

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..27
(D) OTHER INFORMATION: /note= "ANTISENSE TO POSITIONS 927-953 OF SEQ ID NO.: 1."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTGCTGCTGT TGCTGAAGGA GTTGCAT 27

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: SYNTHETIC OLIGONUCLEOTIDE

(ix) FEATURE:
(A) NAME/KEY: misc_feature

(B) LOCATION: 1..21

(D) OTHER INFORMATION: /note= "ANTISENSE TO POSITIONS 916-936 OF SEQ ID NO.: 1."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAGTTGCAT GGTGCTGGCC TCAGCACCA 29

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: SYNTHETIC OLIGONUCLEOTIDE

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 1..21

(D) OTHER INFORMATION: /note= "ANTISENSE TO POSITIONS 927-947 OF SEQ ID NO.: 1."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGTTGCTGA AGGAGTTGCA TAACTCCTT 29

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:
(A) ORGANISM: SYNTHETIC OLIGONUCLEOTIDE

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..25
(D) OTHER INFORMATION: /note= "ANTISENSE TO POSITIONS 611-635 OF SEQ ID NO.: 2."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGCTGTGAA GGTGGTGATT TCCCC 25

CLAIMS

We claim:

1. A method for treating a patient diagnosed as having benign prostatic hyperplasia or a prostatic cancer comprising

administering to said patient a therapeutically effective amount of a composition comprising an antisense oligonucleotide which selectively hybridizes to a gene or mRNA sequence of said patient;

wherein said antisense inhibits expression of said gene or mRNA sequence; and

wherein said gene or mRNA sequence is selected from the group consisting of an AR and an αFGF gene or mRNA sequence.

2. A method as in claim 1 wherein said oligonucleotide is selected from the group consisting of

(a) oligonucleotides comprising at least 10 consecutive bases from SEQ ID NO.: 1;

(b) oligonucleotides comprising at least 10 consecutive bases from a genomic sequence corresponding to SEQ ID NO.: 1; and

(c) oligonucleotides that hybridize to the complements of the oligonucleotides of (a) or (b) under physiological conditions.

3. A method as in claim 1 wherein said oligonucleotide is selected from the group consisting of

(a) oligonucleotides comprising at least 20 consecutive bases from SEQ ID NO.: 1;

(b) oligonucleotides comprising at least 10 consecutive bases from a genomic sequence corresponding to SEQ ID NO.: 1; and

(c) oligonucleotides that hybridize to the complements of the oligonucleotides of (a) or (b) under physiological conditions.

4. A method as in claim 1 wherein said oligonucleotide is selected from the group consisting of

(a) oligonucleotides comprising at least 10 consecutive bases from the group consisting of SEQ ID NO.: 2, SEQ ID NO.: 3 and SEQ ID NO.: 4;

(b) oligonucleotides comprising at least 10 consecutive bases from the joined exons of SEQ ID NO. : 2, SEQ ID NO.: 3 and SEQ ID NO.: 4; and

(c) oligonucleotides that hybridize to the complements of the oligonucleotides of (a) or (b) under physiological conditions.

5. A method as in claim 1 wherein said oligonucleotide is selected from the group consisting of

(a) oligonucleotides comprising at least 20 consecutive bases from the group consisting of SEQ ID NO.: 2, SEQ ID NO.: 3 and SEQ ID NO.: 4;

(b) oligonucleotides comprising at least 20 consecutive bases from the joined exons of SEQ ID NO. : 2, SEQ ID NO.: 3 and SEQ ID NO.: 4; and

(c) oligonucleotides that hybridize to the complements of the oligonucleotides of (a) or (b) under physiological conditions.

6. A method as in claim 1 wherein said oligonucleotide comprises a nucleotide sequence selected from the group consisting of SEQ ID NO.: 5, SEQ ID NO.: 6, SEQ ID NO.: 7, and SEQ ID NO.: 8.

7. A method as in claim 1 wherein said oligonucleotide is a modified oligonucleotide.

8. A method as in claim 7 wherein said oligonucleotide is a modified oligonucleotide including at least one synthetic internucleoside linkage.

9. A method as in claim 8 wherein said synthetic internucleoside linkage is selected from the group consisting of phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate

triesters, acetamidates, and carboxymethyl esters.

10. A method as in claim 7 wherein said oligonucleotide is a modified oligonucleotide having at least one low molecular weight organic group covalently bound to a phosphate group of said oligonucleotide.

11. A method as in claim 7 wherein said oligonucleotide is a modified oligonucleotide having at least one low molecular weight organic group covalently bound to a 2' position of a ribose of said oligonucleotide.

12. A method as in claim 7 wherein said oligonucleotide is a modified oligonucleotide having covalently attached thereto a compound selected from the group consisting of androgen, androgen derivatives, estrogen, estrogen derivatives, estramustine, emcyt and estracyt.

13. A method as in claim 1 wherein said oligonucleotide is administered intravenously at a dosage between 1.0 μg and 100 mg per kg body weight of said patient.

14. A method as in claim 1 wherein said patient has a prostatic cancer which is refractory to anti-androgen or estrogen hormonal therapy.

15. A pharmaceutical composition comprising

a sterile pharmaceutically acceptable carrier; and

a therapeutically effective amount of an isolated antisense oligonucleotide which selectively hybridizes to a gene or mRNA sequence of a patient;

wherein said antisense inhibits expression of said gene or mRNA sequence; and

wherein said gene or mRNA sequence is selected from the group consisting of an AR and an αFGF gene or mRNA sequence.

16. A composition as in claim 15 wherein said oligonucleotide is selected from the group consisting of

(a) oligonucleotides comprising at least 10 consecutive bases from SEQ ID NO.: 1;

(b) oligonucleotides comprising at least 10 consecutive bases from the joined exons of SEQ ID NO.: 1; and

(c) oligonucleotides that hybridize to the complements of the oligonucleotides of (a) or (b) under physiological conditions.

17. A composition as in claim 15 wherein said oligonucleotide is selected from the group consisting of

(a) oligonucleotides comprising at least 20 consecutive bases from SEQ ID NO.: 1;

(b) oligonucleotides comprising at least 20 consecutive bases from the joined exons of SEQ ID NO.: 1; and

(c) oligonucleotides that hybridize to the complements of the oligonucleotides of (a) or (b) under physiological conditions.

18. A composition as in claim 15 wherein said oligonucleotide is selected from the group consisting of

(a) oligonucleotides comprising at least 10 consecutive bases from SEQ ID NO.: 2;

(b) oligonucleotides comprising at least 10 consecutive bases from a genomic sequence corresponding to SEQ ID NO.: 2; and

(c) oligonucleotides that hybridize to the complements of the oligonucleotides of (a) or (b) under physiological conditions.

19. A composition as in claim 15 wherein said oligonucleotide is selected from the group consisting of

(a) oligonucleotides comprising at least 20 consecutive bases from SEQ ID NO.: 2;

(b) oligonucleotides comprising at least 20 consecutive bases from a genomic sequence corresponding to SEQ ID NO.: 2; and

(c) oligonucleotides that hybridize to the complements of the oligonucleotides of (a) or (b) under physiological conditions.

20. A composition as in claim 15 wherein said oligonucleotide comprises a nucleotide

sequence selected from the group consisting of SEQ ID NO.: 5, SEQ ID NO.: 6, SEQ ID NO.: 7, SEQ ID NO.: 8, and SEQ ID NO.: 9.

21. A composition as in claim 15 wherein said oligonucleotide is a modified oligonucleotide.

22. A composition as in claim 15 wherein said oligonucleotide is a modified oligonucleotide including at least one synthetic internucleoside linkage.

23. A composition as in claim 22 wherein said synthetic internucleoside linkage is selected from the group consisting of phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, and carboxymethyl esters.

24. A composition as in claim 21 wherein said oligonucleotide is a modified oligonucleotide having at least one low molecular weight organic group covalently bound to a phosphate group of said oligonucleotide.

25. A composition as in claim 21 wherein said oligonucleotide is a modified oligonucleotide having at least one low molecular weight organic group covalently bound to a 2' position of a ribose of said oligonucleotide.

26. A composition as in claim 21 wherein said oligonucleotide is a modified oligonucleotide having covalently attached thereto a compound selected from the group consisting of androgen, androgen derivatives, estrogen, estrogen derivatives, estramustine, emcyt and estracyt.

27. A pharmaceutical kit comprising the pharmaceutical composition of claim 15 in a pharmaceutically acceptable carrier for intravenous administration.

28. A method for treating a patient diagnosed as having benign prostatic hyperplasia

or a prostatic cancer comprising

administering to said patient a therapeutically effective amount of a composition comprising an antisense oligonucleotide which selectively hybridizes to a gene or mRNA sequence of said patient;

wherein said antisense inhibits expression of said gene or mRNA sequence; and wherein said antisense inhibits or represses prostatic cell growth.

29. A method as in claim 28 wherein said gene is selected from the group consisting of a PSA gene, a probasin gene, an αFGF gene, an androgen receptor gene, an estrogen receptor gene, a telomerase gene, a prohibitin gene, a src gene, a ras gene, a myc gene, a blc-2 gene, a protein kinase-A gene, a plasminogen activator urokinase gene and a methyl transferase gene.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/15081

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07H21/04 A61K31/70 //A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07H A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 05268 A (BAYLOR COLLEGE MEDICINE) 17 March 1994 see page 8, line 14 - page 10, line 20 see example 1 see claims 1,2,17-21,32-35	1,7,13, 15,21, 28,29
X	WO 89 09791 A (UNIV NORTH CAROLINA) 19 October 1989 see page 2, line 12 - line 32 see page 24	1,28,29
X	WO 95 11301 A (UNIV MICHIGAN) 27 April 1995 see claims	28,29

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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1

Date of the actual completion of the international search	Date of mailing of the international search report
14 February 1997	26.02.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Andres, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANCER RESEARCH, (1994 MAY 1) 54 (9) 2372-7., XP002025258 ACHBAROU, A. ET AL.: "Urokinase overproduction results in increased skeletal metastasis by prostate cancer cells in vivo." see the whole document	28,29
A	CANCER SURVEYS, vol. 11, 1991, pages 239-254, XP000616360 SHERIDAN, V. & TEW, K.: "Mechanism based chemotherapy for prostate cancer" cited in the application see the whole document	12,26
O,A	ANTISENSE RES.DEV. 5 (FALL 1995); PAGE 239; ABSTRACT III12, XP002025259 HEAD, M. ET AL.: "Penetration and stability of antisense oligonucleotides injected into the early embryonic chick eye" see abstract & INT.CONF.:'THERAPEUTIC OLIGONUCLEOTIDES FROM CELL TO MAN'; 4 TO 7 APRIL 1995; SEILLAC; FRANCE,	1,4-9
P,X	US 5 556 956 A (ROY ARUN K ET AL) 17 September 1996 see the whole document	1,7-10, 13,15, 21-24, 27-29
P,X	CELL GROWTH AND DIFFERENTIATION, (1996 MAY) 7 (5) 573-86., XP000616505 SHAIN, S. ET AL.: "Endogenous fibroblast growth factor - 1 or fibroblast growth factor -2 modulate prostate cancer cell proliferation." see the whole document	1,4-9, 28,29
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 MAY 31) 271 (22) 13228-33., XP002025260 BOFFA, L. ET AL.: "Invasion of the CAG triplet repeats by a complementary peptide nucleic acid inhibits transcription of the androgen receptor and TATA-binding protein genes and correlates with refolding of an active nucleosome containing a unique AR gene sequence." see the whole document	1-3,7,8, 10,28,29
P,X	WO 96 03875 A (UNIV EMORY) 15 February 1996	28,29
P,A	see page 11, line 12 - page 13, line 21	12,26

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International Application No
PCT/US 96/15081

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,P,X	PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING 37 (0), March 1996, page 344 XP002025261 STEINER, M. ET AL.: "Gene therapy of advanced prostate cancer by in vivo transduction with prostate-targeted antisense c- myc RNA retroviruses." see abstract #2349 & 87TH ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, WASHINGTON, D.C., USA, APRIL 20-24, 1996., -----	28,29

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INTERNATIONAL SEARCH REPORT

Interni al application No.

PCT/US 96/15081

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark : Although claims 1-14, 28-29 (as far as in vivo methods are concerned) are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Info.   ion on patent family members

Internationa' 'plication No
PCT/US 96/15081

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9405268	17-03-94	AU-A-	4846793	29-03-94
WO-A-8909791	19-10-89	EP-A-	0365657	02-05-90
WO-A-9511301	27-04-95	AU-A-	7983294	08-05-95
US-A-5556956	17-09-96	NONE		
WO-A-9603875	15-02-96	AU-A-	3071995	04-03-96

PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/11, C07H 21/04, A61K 31/70 // 48/00

A1

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(71) Applicant (*for all designated States except US*): WORCESTER FOUNDATION FOR BIOMEDICAL RESEARCH [US/US]; 222 Maple Street, Shrewsbury, MA 01545-8000 (US).

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(74) Agent: KINDREGAN, Helen; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

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Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANTISENSE OLIGONUCLEOTIDE CHEMOTHERAPY FOR BENIGN HYPERPLASIA OR CANCER OF THE PROSTATE

(57) Abstract

Methods of selectively inhibiting the growth of or killing prostatic cells, using antisense oligonucleotides to prostate specific genes, are disclosed. The oligonucleotides may have natural nucleic acid structures or may be modified oligonucleotides with enhanced stability or tissue specific targeting. The prostate specific genes to which the antisense may be directed include the AR and the αFGF gene. Pharmaceutical compositions including such antisense oligonucleotides are also described for use in the methods. The methods and products are of particular utility in the treatment of benign prostatic hyperplasia or prostate cancer.

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the antisense oligonucleotides comprise at least 15 consecutive bases, and most preferably, 20-30 consecutive bases from the above-described sequences.

The duplex region, which is preferably at least 4-6 bases joined by a loop of 3-6 bases, stabilizes the oligonucleotide against degradation. These self-stabilized oligonucleotides are easily designed by adding the inverted complement of a 5' or 3' AR or αFGF sequence to the end of the oligonucleotide (see, e.g., Table 1, SEQ ID NO.: 6 and SEQ ID NO.: 7; Tang, J.-Y., et al. (1993) Nucleic Acids Res. 21:2729-2735).

may be composed of "natural" deoxyribonucleotides,

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CAGCTCATGC CCCCTTTCAG ATGTCTTCTG CCTGTTATAA CTCTGCACTA CTCCTCTGCA 3209

GTGCCTTGGG GAATTTCCTC TATTGATGTA CAGTCTGTCA TGAACATGTT CCTGAATTCT 3269

ATTTGCTGGG CTTTTTTTTT CTCTTTCTCT CCTTTCTTTT TCTTCTTCCC TCCCTATCTA 3329

ACCCTCCCAT GGCACCTTCA GACTTTGCTT CCCATTGTGG CTCCTATCTG TGTTTTGAAT 3389

GGTGTTGTAT GCCTTTAAAT CTGTGATGAT CCTCATATGG CCCAGTGTCA AGTTGTGCTT 3449

GTTTACAGCA CTACTCTGTG CCAGCCACAC AAACGTTTAC TTATCTTATG CCACGGGAAG 3509

TTTAGAGAGC TAAGATTATC TGGGGAAATC AAAACAAAAA ACAAGCAAAC AAAAAAAAAA 3569
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1082 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 602..770